NOAA Technical Memorandum NMFS F/NWC-92

Standard Analytical Procedures of the NOAA National Analytical Facility, 1985-1986

Extractable Toxic Organic Compounds, Second Edition

Prepared for
The NOAA National Status and Trends Program

By
William D. MacLeod Jr., Donald W. Brown, Andrew J. Friedman, Douglas G. Burrows, Orlando Maynes, Ronald W. Pearce, Catherine A. Wigren and Richard G. Bogar

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Numerous recent studies demonstrate associations between organic chemical contamination of the aquatic environment and impacts on environmental health and, potentially, on human health (cf. Malins et al. 1984). If the results of one study are to be compared with those of another, uniform analytical methods for the chemicals will be required. To meet this need, NOAA's National Analytical Facility (NAF) prepared this Technical Memorandum as a methods manual for extractable organic chemicals in marine sediments and tissues. It applies specifically to the organic analytes (i.e., the chemicals to be analyzed for) selected for documentation by NOAA's National Status and Trends (S&T) Program.

KEYWORDS: *Organic compounds, *Chemical analysis, *Environmental impacts, *Sediments, *Tissues (Biology), *Toxic substances, *Environmental health, *Water pollution detection.

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STANDARD ANALYTICAL PROCEDURES

OF THE

NOAA NATIONAL ANALYTICAL FACILITY, 1985-86

EXTRACTABLE TOXIC ORGANIC COMPOUNDS

SECOND EDITION

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October 1985

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PREFACE

The analytical procedures for marine environmental samples described herein result from nine years of methods development and application by the National Analytical Facility (NAF) of the National Oceanic and Atmospheric Administration (NOAA). These procedures have been in effect for one year in the National Status and Trends (S&T) Program of NOAA's National Ocean Service. This Second Edition, NOAA Technical Memorandum NMFS F/NWC-90, incorporates additions to and revisions of NOAA Technical Memorandum NMFS F/NWC-64, which is hereby superseded.

Begun in 1984, the S&T Program seeks to document and assess the present status and future trends of environmental quality throughout the nation's coasts and estuaries. Basically, the S&T Program asks:

What are the current conditions of the nation's coastal zone?

Are these conditions getting better or worse?

To answer such questions the S&T Program seeks to employ a nationally uniform set of environmental measurements. To help attain that uniformity, this Technical Memorandum documents the analytical procedures for the extractable toxic organic chemicals.

The National Status and Trends Program consists of the following major components:

- The National Benthic Surveillance Project
- The National "Mussel Watch" Project
- The Water Quality Monitoring Project
- The Synthesis of Historical and New Data

This publication is a laboratory manual for use by analytical chemists working on the first two components. The National Benthic Surveillance Project is conducted by NOAA's National Marine Fisheries Service (NMFS). Under this project NMFS chemists measure toxic chemicals in bottom sediments and in the fish associated with those sediments. Samples come from about 150 coastal and estuarine stations, predominantly in urban, industrial areas, but with nonurban areas included for reference. In the National "Mussel Watch" Project, laboratories under contract to NOAA will analyze mussels and other bivalves for the same toxic chemicals planned for the National Benthic Surveillance Project. These mollusks will come from about 150 coastal and estuarine sites nationwide.

For further information on NOAA's National Status and Trends Program write: NOAA/National Ocean Service, N/OMA32, Rockville, MD 20852.

INTRODUCTION

Numerous recent studies demonstrate associations between organic chemical contamination of the aquatic environment and impacts on environmental health and, potentially, on human health (cf. Malins et al. 1984). If the results of one study are to be compared with those of another, uniform analytical methods for the chemicals will be required. To meet this need, NOAA's National Analytical Facility (NAF) prepared this Technical Memorandum as a methods manual for extractable organic chemicals in marine sediments and tissues. It applies specifically to the organic analytes (i.e., the chemicals to be analyzed for) selected for documentation by NOAA's National Status and Trends (S&T) Program (Table 1).

The analytical procedures for the organic analytes listed in Table 1 are, for a host of reasons, lengthy and complex. Hence, it is important that the laboratories participating in the S&T Program have specific analytical procedures -- procedures that are described in the detail shown here. This manual is primarily for use by analytical chemists of the National Marine Fisheries Service participating in the National Benthic Surveillance Project and by laboratories under contract to NOAA for the National "Mussel Watch" Project. Use in other comparable applications is encouraged, as are suggestions and comments.

NOAA National Analytical Facility

Since its inception in 1976, NOAA's National Analytical Facility has been at the forefront in developing and employing advanced methods to analyze aquatic samples for traces of toxic chemicals. These activities have focused primarily on methods for determining industry-related organic compounds such as aromatic hydrocarbons and chlorinated hydrocarbons in both sediments and organisms. Most of the analytes are listed among the EPA-NRDC "Priority Pollutants" (Environmental Protection Agency 1979).

Over the years NAF methods have found wide application in environmental studies concerning the Strait of Juan de Fuca (MacLeod et al. 1977, Brown et al. 1979), the New York Bight (MacLeod et al. 1981), and Puget Sound (Malins et al. 1980, 1982), among others. As the methods are neither simple nor inexpensive, it is most important that the soundest analytical techniques available be employed and that improvements be continually sought. Thus, evolution of the methodology is assured, and this manual will be updated periodically as methods improve.

Quality of Analytical Data

Horwitz and coworkers (1980) observed that the uncertainty in the analytical results in interlaboratory comparisons increases in a regular progression as the concentrations of the particular analyte descend from fractions of a percent to parts-per-million (ppm) to parts-per-billion (ppb). According to their studies, standard deviations (s) for interlaboratory comparisons of means $(\bar{x})$ around 10 ppb should not be

Table 1. Extractable organic chemicals and internal standards selected for documentation by NOAA's National Status and Trends Program.

aromatic hydrocarbons (AHs)	chlorinated compounds	
naphthalene	hexachlorobenzene	
2-methylnaphthalene	lindane (γ-BHC)	
1-methylnaphthalene	heptachlor	
biphenyl	heptachlor epoxide	
2,6-dimethylnaphthalene	aldrin	
acenaphthene	dieldrin	
fluorene	α-chlordane	
phenanthrene	trans-nonachlor	
anthracene	mirex	
1-methylphenanthrene		
fluoranthene	o,p'-DDE	DDTs
pyrene	p,p'-DDE	
benz[a]anthracene	o,p'-DDD	
chrysene	p,p'-DDD	
benzo[e]pyrene	o,p'-DDT	
benzo[a]pyrene	p,p'-DDT	
perylene		
dibenz[a,h]anthracene	dichlorobiphenyls	PCBs
	trichlorobiphenyls	
	tetrachlorobiphenyls	
internal standards (I-Stds)	pentachlorobiphenyls	
	hexachlorobiphenyls	
naphthalene-d_8	heptachlorobiphenyls	
acenaphthene-d_{10}	octachlorobiphenyls	
perylene-d_{12}	nonachlorobiphenyls	
hexamethylbenzene (HMB)		
tetrachloro-m-xylene (TCMX)	natural product (sewage tracer)	
5α-androstan-3β-ol		
4,4'-dibromooctafluorobiphenyl	coprostanol	

expected to be better than 35% of the grand mean $(\bar{X})$. Our experience (MacLeod et al. 1982) has shown this thesis to be realistic, but it often dismays or confounds statisticians, modelers, and administrators. Nevertheless, the issue must be faced, and the best possible precision must be secured for the analytical results. Accordingly, the methods published here are the best compilation of techniques we could devise.

In implementing these procedures the following quality assurance (QA) protocols have been observed. First, the procedures were validated statistically in NAF's laboratories, consistent with the Horwitz model above. Then, NAF distributed calibrating solutions and previously analyzed sample extracts to the participating laboratories for testing their measuring equipment, i.e., the gas chromatograph(s). Once consistent and satisfactory results have been obtained, interim reference materials (IRMs) are supplied to the laboratories to assess their proficiencies with the overall analytical procedures. This is repeated on a continuing basis throughout the performance of analyses. Our constant goal is to have interlaboratory standard deviations that conform as closely as possible to the Horwitz model.

Summary of Analytical Procedures

In general, analyses of sediment and organisms follow the scheme shown in Figure 1, as summarized below:

> Thaw the sample (if frozen), remove excess water, and weigh the wet sample (ca. 10 g for sediment or 3 g for tissue) to the nearest 0.01 g. Add the extraction solvent (CH_2C1_2) and internal standards (I-Stds), then mix/grind/extract sample with sodium sulfate three times under CH_2Cl_2. Combine the solvent extracts and concentrate them by boiling. Chromatograph the extract concentrate on silica gel and alumina, and collect fractions eluted with pentane (fraction SA1) and 50% CH_2Cl_2 in pentane (fraction SA2). In <u>sediments</u> only, continue to elute with CH_2Cl_2 and CH_3OH (fraction SA3). Concentrate fraction SA2 by boiling, and then chromatograph it on precalibrated Sephadex LH-20. Collect the second fraction from Sephadex chromatography (fraction SA2-L2), and concentrate it to 1 mL if from sediment or to 0.1 mL if from tissue. Analyze fraction SA2-L2 from sediment and tissue (except liver) for the aromatic hydrocarbons (AHs) in Table 1 (page 2) by capillary gas chromatography (GC) with a flame-ionization detector (FID). Also analyze fraction SA2-L2 from all samples for the chlorinated hydrocarbons on page 2, using an electron-capture detector (ECD). If hexachlorobenzene (HCB) is found, also analyze fraction SA1 for HCB as with fraction SA2-L2. Concentrate fraction SA3 from sediments, and analyze it by GC/FID for coprostanol.

The procedures summarized above are presented in detailed sections, each of which deals with a major analytical operation. The order of the sections appears on the Contents page.

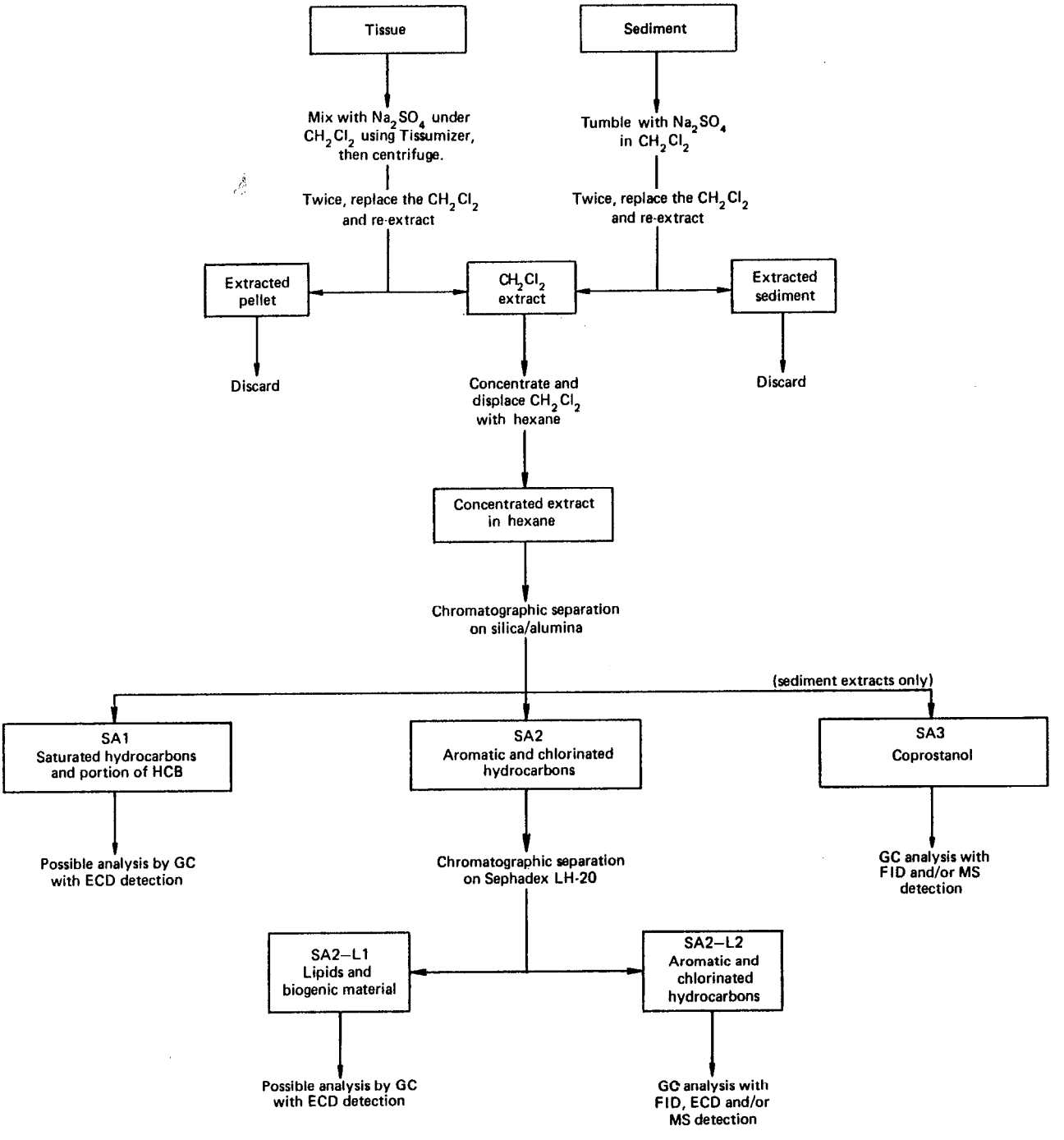

Figure 1. Summary of the analytical procedures of the National Analytical Facility for trace extractable toxic organic analytes.

SECTION 1

MATERIALS

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MATERIALS

Disclaimer: Mention of a product or company name does not imply endorsement by the Department of Commerce to the exclusion of others that may be suitable.

A. Solvents

cyclohexane	Brand: _____, Lot # _____
hexane	Brand: _____, Lot # _____ checked per Section 4E, page 35
methanol (CH_3OH)	Brand: _____, Lot # _____ checked per Section 4A, page 25
redistilled CH_3OH	Prepared from CH_3OH per Section 2, Part C, page 17, and checked per Section 4A, page 25
dichloromethane (CH_2Cl_2)	Brand: _____, Lot # _____ checked per Section 4C, page 31
pentane	Brand: _____, Lot # _____ checked per Section 4D, page 33

B. Column Packings

silica gel	Davison Type 923 or Amicon No. 84080, Lot # _____, checked per Section 5, page 37
alumina	Sigma F-20, 80-200 mesh, Lot # _____, checked per Section 5, page 37
size-exclusion gel	Sephadex LH-20, Lot # _____, checked per Section 6, page 45
sand	Ottawa, MCB, kiln-dried, 30-40 mesh

C. Reagents

Na_2SO_4	Reagent Grade, anhydrous granular
copper	Reagent Grade, fine granular

D. Miscellaneous

boiling chips	Teflon, Norton Chemplast, Chemware

E. Yellow Laboratory Lighting

Yellow fluorescent and/or incandescent lights

Yellow transparent acetate sheeting on windows

F. Standards

HMB GC/I-Std solution, ca. 100 ng/μL of hexamethylbenzene in hexane

Actual conc.: Std #_____, ng/μL _____; Std # _____, ng/μL _____

TCMX GC/I-Std solution, ca. 2 ng/μL of tetrachloro-m-xylene in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

AH I-Std solution, ca. 50 ng/μL of each I-Std in hexane

Actual conc.:	Std # _____	Std # _____
naphthalene-d_8	ng/μL _____	ng/μL _____
acenaphthene-d_{10}	" _____	" _____
perylene-d_{12}	_____	_____

PES I-Std solution, ca. 1 ng/μL of 4,4'-dibromooctafluorobiphenyl in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

COP I-Std solution, ca. 50 ng/μL of 5α-androstan-3β-ol in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

COP GC-calibration-check solution, ca. 5 ng/μL in hexane of:

Actual conc.:	Std # _____	Std # _____
hexamethylbenzene (GC/I-Std)	ng/μL _____	ng/μL _____
5α-androstan-3β-ol	" _____	" _____
coprostanol	" _____	" _____

COP spike solution, ca. 50 ng/uL of coprostanol in hexane

Actual conc.: Std # _____, ng/μL _____; Std # _____, ng/μL _____

F. **Standards (continued)**

AH GC-calibration-check solution, ca. 5 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
hexamethylbenzene (GC/I-Std)	ng/µL _____	ng/µL _____
naphthalene	" _____	" _____
2-methylnaphthalene	_____	_____
1-methylnaphthalene	_____	_____
biphenyl	_____	_____
2,6-dimethylnaphthalene	_____	_____
acenaphthene	_____	_____
fluorene	_____	_____
phenanthrene	_____	_____
anthracene	_____	_____
1-methylphenanthrene	_____	_____
fluoranthene	_____	_____
pyrene	_____	_____
benz[a]anthracene	_____	_____
chrysene	_____	_____
benzo[e]pyrene	_____	_____
benzo[a]pyrene	_____	_____
perylene	_____	_____
dibenz[a,h]anthracene	_____	_____
naphthalene-d_8 (I-Std)	_____	_____
acenaphthene-d_{10} (I-Std)	_____	_____
perylene-d_{12} (I-Std)	_____	_____

F. Standards (continued)

PES GC-calibration-check solution, ca. 0.1 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
tetrachloro-m-xylene (GC/I-Std)	ng/µL _____	ng/µL _____
hexachlorobenzene	" _____	" _____
lindane (γ-BHC)	_____	_____
heptachlor	_____	_____
heptachlor epoxide	_____	_____
aldrin	_____	_____
α-chlordane	_____	_____
trans-nonachlor	_____	_____
dieldrin	_____	_____
mirex	_____	_____
o,p'-DDE	_____	_____
p,p'-DDE	_____	_____
o,p'-DDD	_____	_____
p,p'-DDD	_____	_____
o,p'-DDT	_____	_____
p,p'-DDT	_____	_____
2,4'-dichlorobiphenyl	_____	_____
2,5,4'-trichlorobiphenyl	_____	_____
2,4,2',4'-tetrachlorobiphenyl	_____	_____
2,4,5,2'5'-pentachlorobiphenyl	_____	_____
2,4,5,2',4' 5'- hexachlorobiphenyl	_____	_____
2,3,4,5,6,2',5'-heptachlorobiphenyl	_____	_____
2,3,4,5,2',3',4',5'-octachlorobiphenyl	_____	_____
2,3,4,5,6,2',3',4',5'-nonachlorobiphenyl	_____	_____
4,4'-dibromooctafluorobiphenyl (I-Std)	_____	_____

F. **Standards** (continued)

AH spike solution, ca. 50 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
naphthalene	ng/µL _____	ng/µL _____
2-methylnaphthalene	" _____	" _____
1-methylnaphthalene	_____	_____
biphenyl	_____	_____
2,6-dimethylnaphthalene	_____	_____
acenaphthene	_____	_____
fluorene	_____	_____
phenanthrene	_____	_____
anthracene	_____	_____
1-methylphenanthrene	_____	_____
fluoranthene	_____	_____
pyrene	_____	_____
benz[a]anthracene	_____	_____
chrysene	_____	_____
benzo[e]pyrene	_____	_____
benzo[a]pyrene	_____	_____
perylene	_____	_____
dibenz[a,h]anthracene	_____	_____

F. **Standards (continued)**

PES spike solution, ca. 1 ng/µL in hexane of:

Actual conc.:	Std # _____	Std # _____
hexachlorobenzene	ng/µL _____	ng/µL _____
lindane (γ-BHC)	" _____	" _____
heptachlor	_____	_____
heptachlor epoxide	_____	_____
aldrin	_____	_____
α-chlordane	_____	_____
trans-nonachlor	_____	_____
dieldrin	_____	_____
mirex	_____	_____
o,p'-DDE	_____	_____
p,p'-DDE	_____	_____
o,p'-DDD	_____	_____
p,p'-DDD	_____	_____
o,p'-DDT	_____	_____
p,p'-DDT	_____	_____
2,4'-dichlorobiphenyl	_____	_____
2,5,4'-trichlorobiphenyl	_____	_____
2,4,2',4'-tetrachlorobiphenyl	_____	_____
2,4,5,2',5'-pentachlorobiphenyl	_____	_____
2,4,5,2',4',5'-hexachlorobiphenyl	_____	_____
2,3,4,5,6,2',5'-heptachlorobiphenyl	_____	_____
2,3,4,5,2',3',4',5'-octachlorobiphenyl	_____	_____
2,3,4,5,6,2',3',4',5'-nonachlorobiphenyl	_____	_____

SECTION 2

6:4 CYCLOHEXANE:METHANOL

AZEOTROPE PREPARATION

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6:4 CYCLOHEXANE:METHANOL AZEOTROPE PREPARATION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the solvents.

Glassware

22-L round bottom boiling flask with a 24/40-STJ port, a 71/60-STJ port, and a thermometer well

22-L round bottom receiver flask with a 45/50-STJ port

5-L round bottom receiver flask with a 24/40-STJ port

2-L TC graduated cylinder

200-mm OD, long-stem funnel

adapter, 45/50-STJ to 24/40-STJ

other distillation apparatus (24/40-STJ): fractionation column (5 cm x 50 cm, packed with 7-mm lengths of 6-mm glass tubing), stillhead with 10/30-STJ thermometer port, condenser (Corning 2400 or Kimble 18140), 3-way receiver valve (8-mm bore Teflon stopcock), misc. fittings

Solvents

10 L cyclohexane

8 L methanol

Other Materials and Apparatus

heating mantle for 22-L flask

Variac transformer

2 ea automatic temperature controllers (YSI Models 63RC and 74)

timing clock

Hoffman clamp

boiling chips

2 ea Lab Jax

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Wash all glassware, including the distillation apparatus, twice with CH_2Cl_2 before each run.
2. Attach the 5-L flask to the rear receiver-valve port, and set the receiver valve to collect the distillate in it. Attach the 22-L receiver flask to the front receiver-valve port.
3. Put the 22-L boiling flask in the heating mantle. Place the stillhead into the top of the fractionation column, and fit the column into the 24/40-STJ port of the boiling flask. Align the stillhead outlet and the condenser inlet fittings, then secure the ball and socket joint with the Hoffman clamp.
4. Wash the glass sensor probe of the Model 74 temperature controller twice with CH_2Cl_2, and set it firmly into the 10/30-STJ port at the top of the stillhead. Insert the metal probe of the Model 63RC temperature controller into the thermometer well of the boiling flask.
5. Place a large funnel in the 71/60-STJ port, then fill the boiling flask with 10 L of cyclohexane and 8 L of CH_3OH (i.e., an excess of CH_3OH). Add 40-50 boiling chips, and stopper the flask.
6. Turn on the condenser cooling water. Set the Variac at 60, the Model 74 temperature controller at 55.5°C, and the Model 63RC temperature controller at 68°C. Start the distillation by switching on the timer.
7. Collect 3 L of 6:4 azeotrope forerun (during 6 hr) in the 5-L receiver flask. Then switch the receiver valve to collect most of the distillate in the 22-L receiver during 24 hr.

B. Procedure (continued)

As the solvent temperature in the boiling flask rises toward 65°C, distillation slows. Only a small amount (ca. 1 L), mostly CH_3OH, remains undistilled, so switch the timer off to stop the distillation.

8. Allow the distillation apparatus to cool, then disassemble it. Discard the boiling chips, and set the undistilled solvent aside for recycling (see Note, step 10). Wash the apparatus and flasks twice with CH_2Cl_2. Reassemble as before, pouring only the 6:4 azeotropic distillate (a 2-phase mixture) from the large receiver back into the boiling flask. Add 40-50 boiling chips.

9. Make sure that the cooling water is flowing, then switch timer on, and distill 1 L of forerun into the 5-L flask (see Note, step 10). Switch the receiver valve so as to collect most of the distillate in the 22-L receiver. Distill until the solvent level (ca. 1 L) reaches the bottom of the thermometer well. Switch off the timer.

10. Allow the apparatus to cool. Remove the large receiver. Discard the boiling chips and set the undistilled solvent aside for recycling.

Note: These boiling flask residues and foreruns may be saved and recycled into step 5 of a subsequent distillation. However, they should not be recycled more than twice.

11. Proceed to Section 3 (page 19) with the 6:4 azeotrope.

C. Redistilled Methanol

1. To prepare redistilled CH_3OH, add 1/10th volume of carbon-filtered, distilled H_20 to a volume of azeotrope from step B.7 (page 16).
2. Allow the phases to separate in a separatory funnel.
3. Drain the lower phase into a boiling flask.
4. Adjust the Variac to setting 70, the Model 74 temp. controller to 66°C, and the Model 63RC temp. controller to 75°C.
5. Distill pure CH_3OH through the fractionation column. Check the purity per Section 4A (page 25).

Note: The upper phase remaining in the separatory funnel, mostly cyclohexane, can be recycled through step B.5 (page 16).

SECTION 3

PREPARATION OF 6:4:3 SOLVENT

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PREPARATION OF 6:4:3 SOLVENT

A. Equipment List - Note: CH_2Cl_2-wash all of the glassware and materials contacting the distilled solvent.

Glassware

2-L TC graduated cylinder

200-mm OD, long-stem funnel

20-L carboy

50-mL volumetric pipet

1-L 24/40-STJ Erlenmeyer flask with stopper

4-L standard solvent bottles

Solvents

6:4 cyclohexane:methanol azeotrope (Section 2, step B.11, page 17)

CH_2Cl_2

Other Materials and Apparatus

pipet filler, 3-valve, rubber (for volumetric pipet)

Teflon-lined stopper for carboy

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Prepare a sample of 6:4:3 solvent for purity testing by pipetting 200 mL each of the upper and lower layers of the 6:4 azeotrope into the flask.
2. Add 120 mL of CH_2Cl_2 to the flask and mix well. Check the purity of the solvent by proceeding with this sample to Section 4B (page 29).

B. Procedure (continued)

3. If the purity of the sample from step 2 is acceptable according to Section 4B (page 29), proceed to step 4. Otherwise, return the remaining 6:4 azeotrope to the boiling flask in Section 2, step B.5 (page 16) for redistillation.
4. Transfer the remaining 6:4 azeotrope from step 1 into the carboy in 2000-mL increments, noting the total volume.
5. Multiply the total volume of the 6:4 azeotrope by 0.30. This is the volume of CH_2Cl_2 to be added to the 6:4 azeotrope to make the 6:4:3 solvent.
6. Add the amount of CH_2Cl_2 calculated in step 5 to the carboy.
7. Stopper the carboy, and mix the 6:4:3 solvent until it is completely homogeneous.

a. Transfer the 6:4:3 solvent into 4-L solvent bottles for storage until use in Section 6 (page 45) or in Section 11 (page 77).

SECTION 4

TESTING SOLVENTS FOR PURITY*

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* Criteria: When a solvent sample (except CH_3OH) is analyzed by GC (Section 12, page 85), no GC peaks should occur within 0.1 min of an analyte peak. Moreover, no peaks after the retention time of naphthalene (GC/FID) or tetrachloro-m-xylene (GC/ECD) should give a deflection > 5% on the GC chart. CH_3OH, used solely for washing, should show no GC peaks > 100% of the GC chart after the retention time of naphthalene (GC/FID) or tetrachloro-m-xylene (GC/ECD).

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A. CH_3OH AND REDISTILLED CH_3OH PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample.

Glassware

100-mL TC graduated cylinder

3 ea 500-mL separatory funnels

6 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

transfer pipets (Pasteur style) with rubber bulbs

6 ea 2-mL GC vials

Solvents

200 mL CH_3OH or redistilled CH_3OH (Section 2, Part C, page 18)

135 mL CH_2Cl_2 (not including washes)

1500 mL carbon-filtered, distilled H_20

GC Internal Standards (per sample)

50 µL HMB GC/I-Std solution

50 µL TCMX GC/I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (block contains: Al inserts fitted to the 0.7 mL line of the tube tip, and an Al-foil shroud with TLC plate window, 5 cm taller than tubes in block)

100-µL syringes

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for CH_3OH or Redistilled CH_3OH - Note: Analyze duplicate samples plus a blank

a. Extraction

(1) Add 100 mL of CH_3OH or redistilled CH_3OH (Section 2, Part C, page 18) and 25 mL of CH_2Cl_2 to a separatory funnel. Swirl the funnel for a few seconds to mix well.

(2) Add 250 mL of carbon-filtered, distilled H_20 to the separatory funnel, and shake it vigorously for 2 min. Allow the phases to separate well.

(3) Drain the lower phase into a flask, leaving behind any emulsion layer. Save the contents of the flask.

(4) Add 10 mL of CH_2Cl_2 to the separatory funnel, and shake it vigorously for 2 min. Allow the phases to separate well.

(5) Drain the lower phase into the flask from step 3, including any emulsion layer.

(6) Discard the contents of the separatory funnel.

(7) Pour the extract from the flask back into the separatory funnel.

(8) Wash the flask with 3-4 mL of CH_2Cl_2, and add the washings to the separatory funnel.

(9) Repeat step (8) once. This flask is no longer needed.

(10) Repeat steps (2)-(6), EXCEPT use a fresh flask in step (3), and do not include the emulsion layer in step (5).

b. Concentration

(1) Add 3-4 boiling chips to the flask from step 2.a(10), and attach a Snyder column.

(2) Concentrate the extract in a 60°C water bath to 10-15 mL.

(3) Transfer the extract to a labeled concentrator tube,

2. Procedure for CH_3OH or Redistilled CH_3OH (continued)

b. Concentration

(4) Add a boiling chip to the tube and, using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

(5) Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the extract, and mix on the Vortex Genie for 2 sec at setting 8-10.

(6) Transfer equal amounts of the extract to 2 GC vials, cap the vials, and label them.

(7) Add "R" to label of one of the vials from step (6), and store it as a reserve.

(8) With the other vial from step (6), proceed to GC Analysis (Section 12, page 85).

3. Procedure for Blank

Proceed as in Subsection 2 above, except omit the 100 mL of CH_3OH in step 2.a(1), and perform only a single analysis.

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B. 6:4:3 SOLVENT PURITY

1. Equipment List - Note: CH_2C1_2-wash all glassware and materials contacting the solvent sample.

Glassware

100-mL graduated cylinder

2 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

2 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

6 ea 2-mL GC vials

Solvents

200 mL 6:4:3 solvent (Section 3, step B.2, page 21)

3 mL redistilled CH_3OH

21 mL hexane

GC Internal Standards (per sample)

50 µL HMB GC/I-Std solution

50 µL TCMX GC/I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

100-µL syringes

Vortex Genie

500-mL Teflon wash-bottle (CH_2C1_2-filled)

2. Procedure for 6:4:3 Solvent - Note: Analyze duplicate samples plus a blank

   a. Transfer 100 mL of the 6:4:3 solvent from Section 3, step B.2 (page 21) to a flask.

   b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

   c. Concentrate the sample in a 75°C water bath to 10-15 mL.

   d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

   e. Add a boiling chip and 1 mL of redistilled CH_3OH to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

   f. Add 7 mL of hexane to the tube, and concentrate the sample to > 0.9 mL, < 1.0 mL.

   g. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting 8-10.

   h. Transfer equal amounts of the sample to 2 GC vials, cap the vials, and label them.

   i. Add "R" to the label of one of the vials from step h, and store it as a reserve.

   j. With the other vial from step h, proceed to GC Analysis (Section 12, page 85).

3. Procedure for Blank

   a. Prepare a blank by adding 1 mL of redistilled CH_3OH and 7 mL of hexane to a tube.

   b. Concentrate the solvents to > 0.9 mL, < 1.0 mL.

   c. Proceed as in steps 2.g-2.j above.

c. CH_2Cl_2 PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample.

Glassware

2 ea 500-mL TC graduated cylinders

3 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

6 ea 2-mL GC vials

Solvents

700 mL CH_2Cl_2 from lot to be tested

350 mL CH_2Cl_2 from lot currently in use

GC Internal Standards (per sample)

50 µL HMB GC/I-Std solution

50 µL TCMX GC/I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

100-µL syringes

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for CH_2Cl_2 - Note: Analyze duplicate samples for each lot to be tested, plus a sample of a CH_2Cl_2 lot currently in use.

   a. Add 350 mL of CH_2Cl_2 to a flask.

   b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

   c. Concentrate the sample in a 60°C water bath to 10-15 mL.

   d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

   e. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

   f. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting 8-10.

   g. Transfer equal amounts of the sample to 2 labeled GC vials, cap the vials, and label them.

   h. Add "R" to the label of one of the vials from step g, and store it as a reserve.

   i. With the other vial from step g, proceed to GC Analysis (Section 12, page 85).

D. PENTANE PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample.

Glassware

2 ea 100-mL TC graduated cylinders

3 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3 ea 3-ball 24/40-STJ Snyder columns

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

6 ea 2-mL GC vials

Solvents

200 mL pentane from lot to be tested

100 mL pentane from lot currently in use

GC Internal Standards (per sample)

50 µL HMB GC/I-Std solution

50 µL TCMX GC/I-Std solution

Other Materials and Apparatus

boiling chips

water bath

modified Kontes tube heater (Section 4A, Part 1, page 25)

100-µL syringes

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for Pentane - Note: Analyze duplicate samples for each lot to be tested, plus a sample of a pentane lot currently in use.

a. Add 100 mL of pentane to a flask.

b. Add 3-4 boiling chips, and attach a Snyder column to the flask.

c. Concentrate the sample in a 55°C water bath to 10-15 mL.

d. Transfer the sample to a concentrator tube (no CH_2Cl_2 washes!).

e. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

f. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the sample, and mix on a Vortex Genie for 2 sec at setting 8-10.

g. Transfer equal amounts of the sample to 2 labeled GC vials, cap the vials, and label them.

h. Add "R" to one of the vials from step g, and store it as a reserve.

i. With the other vial from step g, proceed to GC Analysis (Section 12, page 85).

E. HEXANE PURITY

1. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting hexane sample.

Glassware

3 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

6 ea 2-mL GC vials

Solvents

50 mL hexane from lot to be tested

25 mL hexane from lot currently in use

GC Internal Standards (per sample)

50 µL HMB GC/I-Std solution

50 µL TCMX GC/I-Std solution

Other Materials and Apparatus

water bath

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

100-µL syringes

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

2. Procedure for Hexane - Note: Analyze duplicate samples for each lot to be tested, plus a sample of a hexane lot currently in use.

   a. Add 25 mL of hexane to a concentrator tube.

   b. Add a boiling chip to the tube, and using the tube heater, concentrate the sample to > 0.9 mL, < 1.0 mL.

   c. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the sample, and mix on the Vortex Genie for 2 sec at setting 8-10.

   d. Transfer equal amounts of the sample to 2 labeled GC vials, cap the vials, and label them.

   e. Add "R" to the label of one of the vials from step d, and store it as a reserve.

   f. With the other vial from step g, proceed to GC Analysis (Section 12, page 85).

SECTION 5

LOT TESTING/CALIBRATION OF SILICA GEL/ALUMINA

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LOT TESTING/CALIBRATION OF SILICA GEL/ALUMINA

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the silica gel and alumina.

Glassware (per column)

19-mm ID x 30-cm chromatography column with reservoir

2 ea 250-mL beakers

2 ea 500-mL 24/40-STJ Erlenmeyer flasks with stoppers

3-ball 24/40-STJ Snyder column

22 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

22 ea 2-mL GC vials

transfer pipets (Pasteur style) with rubber bulbs

Reagents and Solvents (per column)

20 g silica gel (heated to 700°C for 18 hr, stored at 170°C, and cooled to room temp. in a desiccator just before weighing and use)

10 g alumina (activated at 120°C for 2 hr, then cooled to room temp. in a desiccator just before weighing and use)

7.5 cc activated copper (< 1 hr before use, activate copper by covering it with conc. HCl and stirring with a glass rod, then allowing it to stand for 5 min, followed by washing twice with CH_3OH and then 3x with CH_2Cl_2. Leave the copper covered with CH_2Cl_2 to to avoid contact with air.)

ca. 1 cc sand, acid-washed (steeped in aqua regia (ACS grades HNO_3:HCl, 1:3, v:v) overnight, then washed three times each with H_20, CH_3OH and CH_2Cl_2, dried, and stored at 120°C)

100 mL pentane

200 mL 1:1 CH_2Cl_2:pentane (v:v)

210 mL CH_2Cl_2 (not including washes)

25 mL 10% redistilled CH_3OH in CH_2Cl_2 (v:v)

30 mL 20% redistilled CH_3OH in CH_2Cl_2 (v:v)

A. Equipment List (continued)

GC Internal Standards and Calibration Extracts

50 µL HMB GC/I-Std solution (per SA fraction)

50 µL TCMX GC/I-Std solution (per SA fraction)

silica-gel/alumina calibration extract: Extract 10 samples each of control (relatively clean) sediment and of control mussel, per Sections 7 and 8, respectively. Combine the 20 extracts (2 mL each), and add: (a) 10 mL of AH spike solution, (b) 1 mL of PES spike solution, and (c) coprostanol to give a final concentration of ca. 2 ug/mL (final volume ca. 55 mL).

Other Materials and Apparatus

desiccator

curved-stem funnel (curve glassblown)

powder funnel

2500-µL and 100-µL syringes

glass wool

0.6 x 75-cm glass rod

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

water bath

Vortex Genie

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Column Preparation - Note: Laboratory temp. must be < 80°F (27°C)

1. Prepare the columns just prior to use. On warm days proceed more slowly to avoid vapor bubbles.
2. Fit a 19-mm ID column with a stopcock, add 100 mL of CH_2Cl_2 and a 5-15-mm glass-wool plug. Tamp the plug well to remove any bubbles.
3. Add the alumina to a beaker, and slowly add 20 mL of CH_2Cl_2.

B. Column Preparation (continued)

Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 6.

4. Add the silica gel to a 2nd beaker. Slowly add 40 mL of CH_2Cl_2 to the beaker. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 9.
5. Place a curved-stem funnel into the column reservoir so that the funnel tip hangs well off-center.
6. Swirl the 1st beaker (from step 3) to resuspend the alumina, and pour the slurry into the column.
7. Wash the beaker with ca. 5 mL of CH_2Cl_2, and add the washings to the column. Repeat the wash twice. Place the beaker under the column.
8. After the particles settle, open the stopcock for 30 sec to allow the alumina to pack more tightly, then close the stopcock.
9. Add the silica gel from step 4 to the column, as in steps 6-7 for the alumina.
10. After the silica gel has settled, open the stopcock. While the solvent is still draining, add the sand and then the copper through the powder funnel. Drain to the packing top, then close the stopcock.
11. Add 50 mL of pentane to the column. Drain to the packing top, then close the stopcock. Discard the eluates collected thus far.

C. Column Calibration

1. Using a 2500-µL syringe, place 2 mL of the silica-gel/alumina calibration extract on top of the packing.
2. Place a concentrator tube, labeled "SA1.1", beneath the column.

C. Column Calibration (continued)

3. Open the stopcock, and drain to the packing top, then close the stopcock.
4. From the remaining 50 mL of pentane, add 0.5 mL to the packing. Open the stopcock. Drain to the packing top, then close the stopcock.
5. Repeat step 4 once.
6. Add the rest of the pentane to the column, and elute at ca. 3 mL/min until 20 mL has been collected in the tube. Close the stopcock, and discard the eluate (column dead volume).
7. Place the tube under the column again, and collect 15 mL. Close the stopcock, and set aside the tube for step D.6.
8. Replace the tube with a tube labeled "SA1.2", and collect 2.0 mL. Close the stopcock, and set aside the tube for step D.6.
9. Using tubes labeled successively "SA1.3"-"SA1.11", repeat step 8 9 times, adding 200 mL of 1:1 CH_2Cl_2:pentane (v:v) to the column when the pentane in the column drains to the packing top.
10. Replace the last tube from step 9 with a tube labeled "SA2.1", and collect 20 mL of eluate. Close the stopcock, and set aside the tube for step D.6.
11. Using tubes labeled successively "SA2.2"-"SA2.10", repeat step 10 nine times (9x), adding 50 mL of CH_2Cl_2 when the CH_2Cl_2:pentane in the column drains to the packing top.
12. Replace the last tube in step 11 with a "waste" flask, and drain the remaining solvent to the packing top.
13. Add 25 mL of 10% CH_3OH in CH_2Cl_2 to the column, and drain to the packing top at ca. 2 mL/min. Close the stopcock.

D. Column Calibration (continued)

14. Discard the contents of the waste flask and replace it with a flask labeled "SA3".
15. Add 30 mL of 20% CH_3OH in CH_2Cl_2 to the column and elute all of the solvent into the SA3-labeled flask.

D. Fraction Concentration

1. Add 3-4 boiling chips and attach a Snyder column to the flask from step C.15.
2. Concentrate the fraction in a 70°-75°C water bath to 10-15 mL.
3. Transfer the fraction to a labeled concentrator tube.
4. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube.
5. Repeat step 4 once.
6. Add a boiling chip to each tube from steps C.7-D.5, and using the tube heater, concentrate each fraction to > 0.9 mL, < 1.0 mL.
7. Add 7 mL of hexane to the SA3 fraction and 2 mL of hexane to each remaining tube. Concentrate each fraction to > 0.9 mL, < 1.0 mL.
8. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to each fraction, and mix each on the Vortex Genie for 2 sec at setting 8-10.
9. Transfer each fraction to a labeled GC vial, cap the vial, and proceed to GC Analysis (Section 12, page 85).
10. From the GC analyses, establish the elution volumes for the SA1, SA2 and SA3 fractions, such that: all alkanes elute in the SA1 fraction; coprostanol and androstanol elute in the SA3 fraction; and all other analytes and internal standards present elute in the SA2 fraction.

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SECTION 6

SEPHADEX LH-20 COLUMN PREPARATION AND CALIBRATION

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SEPHADEX LH-20 COLUMN PREPARATION AND CALIBRATION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample.

Glassware (per column calibrated with azulene/perylene)

19-mm ID x 30-cm chromatography column with reservoir

100-mL TC graduated cylinder

Additional Glassware (per column calibrated with sediment/tissue calibration extract)

2 ea 50-mL TC graduated cylinders

500-mL 24/40-STJ Erlenmeyer flask with stopper

22 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

22 ea 2-mL GC vials

transfer pipets (Pasteur style) with rubber bulbs

3-ball 24/40-STJ Snyder column

Reagents and Solvents (per column calibrated with azulene/perylene)

80 cc swelled Sephadex LH-20 (swelled overnight in 6:4:3 solvent), plus 50 mL additional 6:4:3 solvent

2.5 cc sand, acid-washed (Section 5, Part A, page 39)

350 mL 6:4:3 solvent

Additional Reagents and Solvents (per column calibrated with sediment/tissue calibration extract)

200 mL 6:4:3 solvent

hexane and redistilled CH_3OH (as needed)

GC Internal Standards and Calibration Extracts

50 µL HMB GC/I-Std solution (per fraction)

50 µL TCMX GC/I-Std solution (per fraction)

A. Equipment List (continued)

GC Internal Standards and Calibration Solutions

Azulene/perylene calibration solution: Add enough azulene (ca. 10 mg/mL) and perylene (ca. 1 mg/mL) to ca. 50 mL of 6:4:3 solvent to produce a deeply colored solution. Make sure that the azulene and perylene are completely dissolved.

Sediment/tissue calibration extract: Extract 10 samples each of control sediment and of control mussel tissue, per Sections 7 and 8, respectively. Chromatograph these samples-on silica gel/alumina, per Section 10. Combine the 20 SA2 fractions (2 mL each) from Section 10, step E.2, page 75, and add: (a) 1 mL of PES spike solution, and (b) 10 mL of AH spike solution to the combined fractions. Concentrate this to 10 mL, and add sufficient CH_3OH and CH_2Cl_2 to make a 6:4:3 hexane:CH_3OH:CH_2C1_2 solution.

Other Materials and Apparatus

curved-stem funnel (curve glassblown)

glass wool

UV light (UVS·11 Mineralight)

2500-µL and 100-µL syringes

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

waterbath

Vortex Genie

0.6 x 75-cm glass rod

aluminum foil

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Column Preparation

1. Fit a 19-mm ID column with a stopcock, add 10 mL of 6:4:3 solvent and a 5-10-mm glass-wool plug. Tamp the plug to remove any air bubbles.
2. Add ca. 1 cc of sand to the column, and tap the column gently so that the sand forms a smooth layer on top of the glass wool.

B. Column Preparation (continued)

3. Pour the swelled Sephadex gel through the funnel into the column until the gel fills the column and about 1/4 of the reservoir.
4. Allow 10 min for the Sephadex to settle. Open the stopcock, and elute 80 mL of solvent to ensure firm packing. Add more solvent as needed. Leave 30 mL of solvent in the column reservoir. Cover the top with aluminum foil, and allow the packing to settle overnight.
5. Open the stopcock, and elute 10 mL of solvent, then close the stopcock. Remove the excess Sephadex packing from the top with a transfer pipet until the height of the Sephadex is 26.5 cm.
6. Gently add ca. 1 cc of sand onto the packing so that it forms an even layer on the top. e column may be tapped or tilted slightly to get an even layer of sand.)
7. Examine the packing for air bubbles. If bubbles are evident, elute ca. 250 mL of warm (ca. 35°C) solvent through the column. If the bubbles persist, recycle the packing (Part F, page 52).

C. Column Calibration with Azulene/Perylene (All Columns)

1. Place a 100-mL cylinder beneath the column.
2. Using a transfer pipet, cautiously remove any excess 6:4:3 solvent from the top of the packing.
3. Using a transfer pipet, cautiously apply 2 mL of the azulene/perylene calibration solution onto the column. Use a circular motion to dispense the solution just above the packing, and drip the solution slowly down the column wall so as not to disturb the packing.
4. Open the stopcock, drain to the packing top, and close the stopcock.

C. Column Calibration with Azulene/Perylene (All Columns) (continued)

5. Add ca. 0.5 mL of solvent to the top of the column. Drain to the packing top, and close the stopcock.
6. Repeat step 5 once.
7. Add 100 mL of solvent, and open the stopcock.
8. Elute the solvent until all of the perylene has emerged, using the UV light to monitor the perylene. Record the volumes at which the azulene and perylene start and finish eluting.
9. If the azulene emerges in the 50-65 mL range, and the perylene emerges in the 60-80 mL range without distinct tailing on the packing, proceed to step 10. Otherwise, recycle the packing (Part F, page 52).
10. Discard the eluate. Add 50 mL of solvent to the column, and flush the packing by eluting 50 mL into the cylinder. Again, discard the eluate.
11. The column is now ready for the next sample.

Note: If the column is to be stored, maintain 30-50 mL of solvent in the column reservoir, and cover the top with aluminum foil. Remove the solvent if it separates into 2 phases, add 80 mL of fresh solvent, and elute 50 mL.

D. Column Calibration with Sediment/Tissue Calibration Extract

1. Set aside one representative column for every 10 columns made. Remove any excess 6:4:3 solvent with a transfer pipet.
2. Wash the column tip with CH_2Cl_2, and place a 50-mL cylinder under the column.
3. Using a transfer pipet, cautiously apply 2 mL of sediment/tissue calibration extract onto the column. Use a circular motion to dispense the extract just above the the packing, dripping it slowly down the column wall so as not to disturb the packing. Drain to the packing top, and close the stopcock.

D. Column Calibration with Calibration Extract (continued)

4. Add ca. 0.5 mL of solvent to the column. Drain to the packing top, and close the stopcock. Repeat this step once.
5. Add 200 mL of solvent to the column, and collect 25 mL of eluate in the cylinder. Close the stopcock, and discard the eluate.
6. Place a concentrator tube labeled "L1.0" under the column, and collect 5.0 mL of eluate. Close the stopcock, and set aside the tube for step E.3
7. Place a concentrator tube labeled "L1.1" under the column, and collect 1.0 mL of eluate. Close the stopcock, and set aside the the tube for step E.3.
8. Repeat step 7 fourteen times (14x), labeling the successive fractions "L1.2" through "L1.15".
9. Replace the last tube with a 50-mL cylinder labeled "L2.0", and collect 50 mL of eluate. Close the stopcock, and transfer the eluate to a flask labeled "L2.0".
10. Wash down the cylinder with 3-4 mL of CH_2Cl_2, and add the washings to the flask. Repeat this step once.
11. Set the flask aside for step E.1 and the cylinder for step 14.
12. Place a concentrator tube labeled "L2.1" under the column, and collect 10 mL of eluate. Close the stopcock, and set aside the tube for step E.3.
13. Repeat step 12 four times (4x), labeling the successive fractions "L2.2" through "L2.5".
14. Replace tube "L2.5" with the cylinder from step 11, and flush the packing by eluting 50 mL of solvent. Discard this eluate.

E. Fraction Concentration

1. Add 3-4 boiling chips to the flask from step D.11, attach a Snyder column, and concentrate the fraction in a 75°C water bath to 10-15 mL.
2. Transfer fraction L2.0 to a labeled concentrator tube. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the flask. Repeat the wash once.
3. Add 1 mL of CH_3OH and a boiling chip to each tube from steps D.7-E2. Using the tube heater, concentrate each fraction to > 0.9 mL, < 1.0 mL.
4. Add 7 mL of hexane to each tube, and concentrate to > 0.9 mL, < 1.0 mL.
5. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to each fraction.
6. Mix each fraction on the Vortex Genie for 2 sec at setting 8-10.
7. Transfer each fraction to a labeled GC vial and proceed to GC Analysis (Section 12, page 85).
8. Verify by GC analysis that the analytes are separated from lipids. Establish the elution volumes so as to leave all analytes and internal standards present in the L2 fraction.

F. Recycle of Column Packing - Note: This is an optional procedure which requires additional equipment as described below

Summary: Decant any solvent in the column reservoir. Empty the column packing into a beaker 4 times the volume of the packing. Wash with CH_2Cl_2. Add enough CH_2Cl_2 to float the Sephadex particles in the upper half of the beaker. Remove all glass wool with forceps (mandatory). Cover the beaker and let it stand for 1-2 hr. Decant the floating particles into a fritted-glass funnel attached to an aspirator, leaving the sand in the beaker. Aspirate the CH_2Cl_2 from the Sephadex particles, and set them aside. Swell these particles overnight in 6:4:3 solvent before reusing.

SECTION 7

SEDIMENT EXTRACTION

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SEDIMENT EXTRACTION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample or extract.

Glassware (per sample)

250-mL tumbler/centrifuge bottle (amber, Boston round) with Teflon cap (Savillex, 24-mm)

500-mL 24/40-STJ Erlenmeyer flask with stopper

25-mL 19/22-STJ Kontes concentrator tube with stopper

3-ball 24/40-STJ Snyder column

powder funnel (use more than 1, if needed)

Solvents and Reagents (per sample)

300 mL CH_2Cl_2 (not including washes)

50 g Na_2SO_4 (CH_2Cl_2-washed, dried, stored at 120°C, and cooled to room temp. in a desiccator before weighing and use)

hexane (as needed)

Internal Standards and Spike Solutions

AH I-Std, PES I-Std, COP I-Std, AH spike, PES spike, and COP spike solutions

Other Materials and Apparatus

1 spatula per sample

modified rock tumbler (Model NF-1, Lortone Inc., 2856 NW Market St., Seattle, WA 98107; belt guard is removed)

centrifuge (to accommodate the tumbler/centrifuge bottles)

masking tape

desiccator

boiling chips

1000-µL and 100-µL syringes

A. Equipment List (continued)

4 ea 2-mL GC vials

modified Kontes tube heater (Section 4A, Part 1, page 25)

water bath

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Sample Extraction

1. Decant the excess water from the sediment, and stir it to homogenize. Discard all pebbles, seaweed, wood, crabs, etc.
2. Using a spatula and powder funnel, weigh 10 ± 0.5 g of sediment to the nearest 0.01 g into a tared bottle. Record the weight in the log book.
3. Set aside ca. 10 g of the homogenized sediment for the Dry Weight Determination (Section 9, page 65). Store the remaining sample in a freezer.
4. Centrifuge each sample bottle at $\leq$ 1500 rpm for 5 min. Decant and discard the H_2O.
5. To each sediment sample add: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, and (d) 100 µL of COP I-Std solution. Make certain that the solutions are placed into the CH_2Cl_2.
6. For each set of samples prepare a spiked blank ("reagent spike") by adding to an empty bottle: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, (d) 100 µL of COP I-Std solution, (e) 100 µL of PES spike solution, (f) 100 µL of AH spike solution, and (g) 100 µL of COP spike solution.

B. Sample Extraction (continued)

7. If the sample set requires a field blank ("sediment blank"), prepare this by washing down the empty sediment sample container 3 times with 10 mL of CH_2Cl_2 each time and adding the combined washings to a bottle. Add 70 mL more of CH_2Cl_2 to the bottle and proceed as in the next step starting at (b).

8. For each set of samples prepare a blank ("reagent blank") by adding to an empty bottle: (a) 100 mL of CH_2Cl_2, (b) 100 µL of AH I-Std solution, (c) 100 µL of PES I-Std solution, and (d) 100 µL of COP I-Std solution.

9. Prepare 2 AH/PES analyte-calibration solutions (for Section 11, step F.4, page 83) by adding to each of 2 vials: (a) 600 µL of hexane, (b) 100 µL of AH spike solution, (c) 100 µL of AH I-Std solution, (d) 100 µL of PES I-Std solution, and {e) 100 µL of PES spike solution.

10. Prepare 2 COP analyte-calibration solutions (for Section 10, step G.4, page 76) by adding to each of 2 vials (a) 800 µL of hexane, (b) 100 µL of COP I-Std solution, and (c) 100 µL of COP spike solution.

11. Add 50 g of Na_2SO_4 to each bottle in steps 5-8.

12. Screw each bottle cap on just tight enough to prevent leakage.

    Note: Do not overtighten so as to deform the cap and cause leakage.

13. Tape the cap to the bottle crosswise over the top with 2 strips of masking tape.

14. Manually shake each bottle until the contents are loose, then roll for 16 hr (i.e., overnight) on the tumbler at 100-250 rpm.

B. Sample Extraction (continued)

15. Remove the tape from each bottle. If the sample does not immediately settle, centrifuge it at < 1500 rpm for 5 min.
16. Decant each extract into a labeled flask.
17. Add 100 mL of CH_2Cl_2 to each sample, and repeat steps 12-15, except roll each bottle for 6 hr (i.e., during the day).
18. Decant the 2nd extract into the flask from step 16.
19. Repeat step 17, except roll each bottle for 16 hr (i.e., overnight).
20. Add the 3rd extract from step 19 to the flask from step 18.

C. Extract Concentration

1. Add 3-4 boiling chips to the flask containing the CH_2Cl_2 extract from step B.20, and attach a Snyder column.
2. Concentrate the extract in a 60°C water bath to 10-15 mL, and transfer it to a labeled concentrator tube.
3. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube.
4. Repeat step 3 once.
5. Add a boiling chip to the tube, and using the tube heater, concentrate the extract to $\geq$ 0.9 mL, < 1.0 mL.
6. Add 3 mL of hexane to the tube, and concentrate the extract to 2 mL on the tube heater.
7. Proceed to Silica Gel/Alumina Chromatography (Section 10, page 69).

SECTION 8

TISSUE EXTRACTION

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TISSUE EXTRACTION

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample or extract.

Glassware (per sample)

100-mL centrifuge tube with Teflon-lined cap

500-mL 24/40-STJ Erlenmeyer flask with stopper

25-mL 19/22-STJ Kontes concentrator tube with stopper

3-ball 24/40-STJ Snyder column

Solvents and Reagents (per sample)

80 mL CH_2Cl_2 (not including washes)

hexane (as needed)

25 g Na_2SO_4 (CH_2Cl_2-washed, dried, stored at 120°C, and cooled to room temp. in a desiccator just before weighing and use)

Internal Standards and Spike Solutions

AH I-Std, PES I-Std, AH spike, and PES spike solutions

Other Materials and Apparatus

1 spatula per sample

Tekmar Tissumizer

desiccator

centrifuge (to accommodate the 100-mL centrifuge tubes)

boiling chips

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

1000-µL and 100-µL syringes

2 ea 2-mL GC vials

water bath

modified Kontes tube heater (Section 4A, Part 1, page 25)

Vortex Genie

Teflon sheeting (to line centrifuge bottle caps)

B. Sample Extraction

1. Using a spatula, and being careful to place the sample on the bottom and not the sides, weigh 3 + 0.5 g of sample to the nearest 0.01 g into the centrifuge tube. Set aside ca. 1 g for Dry Weight Determination (Section 9, page 65). Record the weight in the log book.
2. Store the remaining sample in a freezer.
3. To each tissue sample in a centrifuge tube add: (a) 35 mL of CH_2Cl_2, (b) 20 µL of AH I-Std solution, and (c) 20 µL of PES I-Std solution. Make certain that the solutions are placed into the CH_2Cl_2.
4. For each set of samples prepare a spiked blank ("reagent spike") by adding to a centrifuge tube containing 35 mL of CH_2Cl_2: (a) 20 µL of AH I-Std solution, (b) 20 µL of PES I-Std solution, (c) 20 µL of AH spike solution, and (d) 20 µL of PES spike solution.
5. If the sample set requires a field blank ("tissue blank"), prepare this by washing down the empty sample container 3 times with 10 mL of CH_2Cl_2 each time and adding the combined washings to an empty centrifuge tube. Add 5 mL more of CH_2Cl_2 to the tube, and proceed as in the next step starting at (b).
6. For each set of samples prepare a blank ("reagent blank") by adding to an empty centrifuge tube: (a) 35 mL of CH_2Cl_2, (b) 20 µL of AH I-Std solution, and (c) 20 µL of PES I-Std solution.
7. Prepare 2 AH/PES analyte-calibration solutions (for Section 11, step G.5, page 83) by adding to each of 2 vials: (a) 900 µL of hexane, (b) 20 µL of AH I-Std solution, (c) 20 µL of PES I-Std solution, (d) 20 µL of AH spike solution, and (e) 20 µL of PES spike solution.

B. Sample Extraction (continued)

8. Add 25 g of Na_2SO_4 to each tube from steps 3-6.
9. Macerate/extract the sample in the tube for 1 min with the Tissumizer at setting 100. Then continue at setting 50 for 2 min. Avoid spattering the tissue.
10. Wash down the probe with CH_2Cl_2, collecting the washings in the centrifuge tube.
11. Centrifuge the sample for 5 min at < 2000 rpm.
12. Decant the extract into a labeled flask.
13. Add 35 mL of CH_2Cl_2 to the tube.
14. Repeat steps 9-12 once.
15. Wash the Na_2SO_4/sample mass by adding 10 mL of CH_2Cl_2 to the tube, and mixing on the Vortex Genie for 5-10 seconds at setting 5-6.
16. Repeat steps 11-12 once.

c. Concentration of Extract

1. Add 3-4 boiling chips, and attach a Snyder column to the flask containing the CH_2Cl_2 extract from step B.16.
2. Concentrate the extract in a 60°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube.
4. Repeat step 3 once.
5. Add a boiling chip to the tube, and using the tube heater, concentrate the extract to > 0.9 mL, < 1.0 mL.
6. Add 3 mL of hexane to the tube, and concentrate the extract to 2 mL on the tube heater.
7. Proceed to Silica Gel/Alumina Chromatography (Section 10, page 69).

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SECTION 9

DRY WEIGHT DETERMINATION

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DRY WEIGHT DETERMINATION

A. Equipment List

analytical balances (requirements in steps B.4 and C.3)

spatula(s)

aluminum weighing pan(s)

aluminum foil, 12-inch width

drying oven (120°C)

desiccator

forceps

B. Sediment Procedure

1. Etch the sample number on the tab of the weighing pan.
2. Place up to 3 aluminum pans on 1/2 of a 9-inch strip of aluminum foil. Fold the aluminum foil over the weighing pan(s) to form an envelope. Close the envelope, but do not seal it, then place it in the drying oven overnight.
3. Cool the envelope containing the pan in a desiccator for 30 min.
4. Remove the pan from the envelope, and weigh the pan to the nearest 0.01 g. Record the pan weight as the Tare Weight in the log book.
5. Stir the sediment with a spatula to homogenize it, and discard all pebbles, biota, detritus, etc.
6. Add 10 ± 0.5 g of the sediment to the pan.
7. Record the weight to the nearest 0.01 g in the log book as the Wet Weight.
8. Return the weighing pan to the foil envelope, and close the envelope, but do not seal it.
9. Dry the sample in the drying oven for 24 hr.
10. Remove the sample from the oven, and cool it in the desiccator for 30 min.

B. Sediment Procedure (continued)

11. Reweigh the sample, and record the dry weight to the nearest 0.01 g in the log book as the Dry Weight.

C. Tissue Procedure

1. Proceed as in steps B.l-B.4, except use forceps to handle the pan and weigh the pan to the nearest 0.1 mg.
2. With a spatula, spread ca. 0.5 g of tissue onto the pan.
3. Record the weight to the nearest 0.1 mg in the log book as the Wet Weight.
4. Proceed as in steps B.8-B.11, except use the forceps to handle the pan and record the weight to the nearest 0.1 mg.

D. Dry Weight Calculation

1. Calculate Dry Wt % as follows:

$$\text{Dry Wt \%} = \frac{\text{Dry Weight - Tare Weight}}{\text{Wet Weight - Tare Weight}} \times 100.$$

SECTION 10

SILICA GEL/ALUMINA CHROMATOGRAPHY

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SILICA GEL/ALUMINA CHROMATOGRAPHY

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the extract or fractions.

Glassware (per sample)

19-mm ID x 30-cm chromatography column with reservoir

2 ea 250-mL beakers

50-mL TC graduated cylinder

500-mL 24/40-STJ Erlenmeyer flask with stopper*

2 ea* 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

2 ea* transfer pipets (Pasteur style) with rubber bulbs

3-ball 24/40-STJ Snyder column*

2-mL GC vial*

Reagents and Solvents (per sample)

20 g silica gel (heated to 700°C for 18 hr, stored at 170°C, and cooled to room temp. in a desiccator just before weighing and use)

10 g alumina (activated at 120°C for 2 hr, then cooled to room temp. in a desiccator just before weighing and use)

160/210 mL CH_2Cl_2 for tissue/sediment, resp. (not including washes)

25 mL 10% redistilled CH_3OH in CH_2Cl_2 (v:v), for sediment only

30 mL 20% redistilled CH_3OH in CH_2Cl_2 (v:v), for sediment only

50 mL pentane t the amount calibrated in Section 5 to elute the column dead volume + the SA1 fraction

mL of 1:1 (v:v) CH_2Cl_2:pentane (the amount calibrated in Section 5 to elute the SA2 fraction)

hexane, CH_2Cl_2 and redistilled CH_3OH, as needed

ca. 1 cc sand, acid washed (Section 5, Part A, page 39)

7.5 cc activated copper (Section 5, Part A, page 39), for sediment only

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* Add 1 ea for sediment samples

A. Equipment List (continued)

GC Internal Standards

50 µL HMB GC/I-Std solution per SA3 fraction

10 µL TCMX GC/I-Std solution per SA1 fraction

Other Materials and Apparatus

desiccator

powder funnel

curved-stem funnel (curve glassblown)

0.6 x 75-cm glass rod

glass wool

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

boiling chips

modified Kontes tube heater (Section 4A, Part 1, page 25)

water bath

Vortex Genie

100-µL and 10-µL syringes

B. Column Preparation - Note: The laboratory temp. must be < 80°F (27°C)

1. Prepare the columns just prior to use. On warm days proceed more slowly to avoid vapor bubbles.
2. Fit a 19-mm ID column with a stopcock, add 100 mL of CH_2Cl_2 and a 5-15-mm glass-wool plug. Tamp the plug well to remove any bubbles.
3. Add the alumina to a beaker, and slowly add 20 mL of CH_2Cl_2. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 6.
4. Add the silica gel to a 2nd beaker. Slowly add 40 mL of CH_2Cl_2 to the beaker.

B. Column Preparation (continued)

5. Gently swirl the beaker for 30 sec, and let it stand for 5 min (to remove all air bubbles) until used in step 10.
6. Place a curved-stem funnel into the column reservoir so that the funnel tip hangs well off-center.
7. Swirl the beaker to resuspend the alumina from step 3, and pour the slurry into the column.

a. Wash the beaker with ca. 5 mL of CH_2Cl_2, and add the washings to the column. Repeat the wash twice, then place the beaker under the column tip.

9. After the particles settle, open the stopcock for 30 sec to allow the alumina to pack more tightly, then close the stopcock.
10. Add the silica gel from step 4 to the column, as in steps 7-8 for the alumina.
11. After the particles settle, open the stopcock. While the solvent still drains, add the sand through the powder funnel (for sediments: then add the copper). Drain to the packing top, then close the stopcock.
12. Add 50 mL of pentane to the column. Drain to the packing top, then close the stopcock. Discard the eluates collected thus far.

c. Chromatography of Extract

1. Wash the column tip with CH_2Cl_2, remove the waste beaker from beneath the column, and replace it with a cylinder.
2. With a transfer pipet, cautiously transfer the sediment extract (Section 7, step C.6, page 58) or the tissue extract (Section 8, step C.6, page 63) to the top of the packing. Drain to the packing top, then close the stopcock.

c. Chromatography of Extract (continued)

3. From the remaining pentane, wash down the tube that contained the extract with 0.5 mL, and add the washings to the top of the packing. Drain to the packing top, then close the stopcock.
4. Repeat step 3 once.
5. Wash down the tube with ca. 0.5 mL of the 1:1 CH_2Cl_2:pentane, and hold the washings in the tube for step 13.
6. From the remainder of the pentane, add ca. 2 mL to wash down the column wall. Drain to the packing top, then close the stopcock.
7. Repeat step 6 once.
a. Add the rest of the pentane, and continue eluting at ca. 3 mL/min.
9. Collect 20 mL of eluate, then close the stopcock, and discard the contents of the cylinder.
10. Replace the cylinder with a concentrator tube labeled "SA1". Partially open the stopcock and continue eluting until __ mL have been collected (the amount calibrated in Section 5 for fraction SA1), then close the stopcock.
11. Set aside the SA1-labeled tube for step E.1, page 75.
12. Place a flask labeled "SA2" under the column. Drain to the packing top, then close the stopcock.
13. Add the washings from the tube (set aside in step 5) to the top of the packing. Drain to the packing top, then close the stopcock.
14. Wash down the tube with 0.5 mL of the 1:1 CH_2Cl_2:pentane, and add the washings to the top of the packing. Drain to the packing top, then close the stopcock.
15. Add the remaining 1:1 CH_2Cl_2:pentane to the column, and partially open the stopcock. Drain to the packing top and close the stopcock.
16. Set aside the SA2-labeled flask for step F.1, page 75.

D. For Sediment Only

1. Place a "waste" flask under the column, and add 50 mL of CH_2Cl_2 to the column. Drain to the packing top, and close the stopcock.
2. Add 25 mL of 10% CH_3OH in CH_2Cl_2 to the column. Drain to the packing top at ca. 2 mL/min, and close the stopcock.
3. Discard the contents of the waste flask, and replace the flask with one labeled "SA3".
4. Add 30 mL of 20% CH_3OH in CH_2Cl_2 to the column. Elute all of the solvent into the SA3-labeled flask, and set it aside for step G.1, page 76.

E. Concentration of Fraction SA1

1. Add a boiling chip to the tube from step C.11, and using the tube heater, concentrate the SA1 fraction to > 0.9 mL, < 1.0 mL.
2. Add 2 mL of hexane to the tube, and concentrate the fraction to ≥ 0.9 mL, < 1.0 mL.
3. Add 10 µL of TCMX GC/I-Std solution to the tube, and mix for 2 set on the Vortex Genie at setting 8-10.
4. Transfer the concentrate into a GC vial, label it as "SA1", cap the vial, and store it in the freezer until needed.

F. Concentration of Fraction SA2

1. Add 3-4 boiling chips to the SA2-labeled flask from step C.16, and attach a Snyder column.
2. Concentrate the SA2 fraction in a 60°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube. Repeat this step once.

F. Concentration of Fraction SA2 (continued)

4. Continue concentrating the SA2 fraction in the same manner as the SA1 (steps E.1-E.2).
5. Add appropriate amounts of CH_3OH and CH_2Cl_2 to make ≤ 2.3 mL of a solution of 6:4:3 hexane:CH_3OH:CH_2Cl_2 (v:v:v).
6. Proceed to Sephadex LH-20 Chromatography (Section 11, page 77).

G. Concentration of Fraction SA3 (Sediment Only)

1. Concentrate the fraction in the SA3-labeled flask from step D.4 in the same manner as for Fraction SA2 (steps F.1-F.4), except use a 75° C bath in step 1, and add 7 mL of hexane in step 2.
2. Add 50 µL of HMB GC/I-Std solution to the tube, and mix for 2 set on the Vortex Genie at setting 8-10.
3. Transfer the concentrate into a GC vial, label it as "SA3", and cap the vial.
4. Add 50 µL of HMB GC/I-Std solution to the COP analyte-calibration solution vials from Section 7, step B.10 (page 57).
5. Proceed to GC Analysis (Section 12, page 85), and analyze for coprostanol.

SECTION 11

6:4:3 SEPHADEX LH-20 CHROMATOGRAPHY

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6:4:3 SEPHADEX LH-20 CHROMATOGRAPHY

A. Equipment List - Note: CH_2Cl_2-wash all glassware and materials contacting the extract or fractions.

Glassware (per sample)

50-mL TC graduated cylinder

100-mL TC graduated cylinder

500-mL 24/40-STJ Erlenmeyer flask with stopper

3-ball 24/40-STJ Snyder column

2 ea 25-mL 19/22-STJ Kontes concentrator tubes with stoppers

transfer pipets (Pasteur style) with rubber bulbs

3 ea 2-mL GC vials (substitute 1 conical vial for tissue extract)

Solvents (per sample)

200 mL 6:4:3 cyclohexane:CH_3OH:CH_2Cl_2 solution (v:v:v)

2 mL redistilled CH_3OH

GC Internal Standards (per SA2-L2 or SA2-L1 fraction)

HMB GC/I-Std solution: 50 µL (sediment L2); 10 µL (tissue L2)

TCMX GC/I-Std solution: 50/10 µL (sediment L2/L1); 10/10 µL (tissue L2/L1)

Other Materials and Apparatus

aluminum foil

activated copper (Section 5, Part A, page 39)

Vortex Genie

calibrated Sephadex LH-20 columns (Section 6, page 45)

boiling chips

water bath

modified Kontes tube heater (Section 4A, Part 1, page 25)

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

syringes: 100-µL (sediment); 10-µL (tissue)

B. Special Instructions

1. The extract must be dissolved in the solvent (no layers), with the total volume ≤ 2.3 mL.
2. The fraction volumes are dependent on the column calibration. Occasionally check the column calibration (Section 6, page 45).
3. When removing or adding solvent (or extract), extreme care must be used to avoid disturbing the column packing.
4. During column storage, maintain 30-50 mL of the solvent in the column reservoir and cover the top with aluminum foil to minimize evaporation. If the solvent in the reservoir separates into 2 phases, remove it and replace it with > 80 mL of fresh 6:4:3 solvent, then e-lute 50 mL.

c. Chromatography

1. Remove the excess solvent from the top of the column using a transfer pipet.
2. Add 10 mL of the 6:4:3 solvent to the column. Drain to the packing top, and close the stopcock. Discard the eluate.
3. Wash the column tip with CH_2Cl_2, and place the 50-mL cylinder under the column.
4. Using a transfer pipet, carefully apply the 2-mL extract from Section 10, step F.5 (page 76) to the column. Use a circular motion to dispense the sample immediately above the packing, dripping it slowly down the column wall so as not to disturb the packing.
5. Drain to the packing top, and close the stopcock.
6. Wash down the tube with 0.5 mL of the solvent, and apply the washings to the column. Drain to the packing top, and close the stopcock.

C. Chromatography (continued)

7. Repeat step 6 once.
8. Wash down the column wall with ca. 3 mL of the solvent, applied above the base of the reservoir. Drain to the packing top, and close the stopcock.
9. Repeat step 8 once.
10. Cautiously add ca. 150 mL of the solvent to the column (add more as needed) without disturbing the packing.
11. Collect 25 mL of eluate in the 50-mL cylinder. Close the stopcock, and discard this eluate.
12. Replace the cylinder with a concentrator tube labeled "SA2-L1". Open the stopcock, collect ___ mL of eluate (the amount calibrated in Section 6 for fraction SA2-L1), then close the stopcock.
13. Set aside the SA2-Ll-labeled tube for step D.l, page 82.
14. Place the 100-mL cylinder labeled "SA2-L2" under the column. Open the stopcock, and collect ___ mL of eluate (the amount calibrated in Section 6 for fraction SA2-L2). Close the stopcock, and transfer the eluate to a flask labeled "SA2-L2".
15. Wash down the cylinder with 3-4 mL of CH_2Cl_2, and add the washings to the flask.
16. Repeat step 15 once, and set the flask aside for step E.1, page 82.
17. Replace the 100-mL cylinder with a waste cylinder, and elute 50 mL of solvent to flush the column. Discard this eluate.
18. The column is now ready for the next sample.

D. Concentration of Fraction SA2-L1

1. Add 1 mL of CH_3OH and a boiling chip to the tube from step C.13, and using the tube heater, concentrate the SA2-L1 fraction to > 0.9 mL, < 1.0 mL.
2. Add 7 mL of hexane to the tube, and concentrate the fraction to ≥ 0.9 mL, < 1.0 mL.
3. Add 10 µL of TCMX GC/I-Std solution to the tube, and mix for 2 sec on the Vortex Genie at setting 8-10.
4. Transfer the fraction to a GC vial, cap the vial, label it, and store it in the freezer until needed.

E. Concentration of Fraction SA2-L2

1. Add 3-4 boiling chips to the flask from step C.16, and attach a Snyder column.
2. Concentrate the SA2-L2 fraction in a 75°C water bath to 10-15 mL, and transfer it to a concentrator tube.
3. Wash down the flask with 3-4 mL of CH_2Cl_2, and add the washings to the tube. Repeat this step once.
4. Continue concentrating the SA2-L2 fraction in the same manner as the SA2-L1 (steps D.1-D.2).
5. Proceed to step F.1 for sediment or step G.1 for tissue.

F. Fraction SA2-L2 from Sediment

1. Add a few grains of activated copper to the tube from step E.5 until no further discoloring occurs, then stopper the tube and and let it stand overnight in a refrigerator.
2. Add 50 µL of HMB GC/I-Std solution and 50 µL of TCMX GC/I-Std solution to the tube. Mix on the Vortex Genie for 2 sec at setting 8-10.

F. Fraction SA2-L2 from Sediment (continued)

3. Transfer equal amounts of the fraction to 2 GC vials, cap the vials, and label them.
4. Add 50 µL of HMB GC/I-Std solution to the AH/PES analyte-calibration solution vials from Section 7, step B.9 (page 57). Similarly, add 50 µL of TCMX GC/I-Std solution to these vials.
5. Add "R" to the label of one of the vials from step 3, and store it in a freezer as a reserve. Proceed with the other vials from steps 3 and 4 to GC analysis (Section 12, page 85).

G. Fraction SA2-L2 from Tissue, EXCEPT Contaminated Livers (see Part H)

1. Add 10 µL of HMB GC/I-Std solution and 10 µL of TCMX GC/I-Std solution to the tube from step E.5 (page 82).
2. Mix on the Vortex Genie for 2 sec at setting 8-10.
3. Transfer 1/2 of the fraction to a GC vial, cap the vial, and label it.
4. Add "R" to the vial label, and store the vial in a freezer as a reserve. Set aside the tube for step 7.
5. Add 10 µL of HMB GC/I-Std solution and 10 µL of TCMX GC/I-Std solution to the analyte-calibration solution vials from Section 8, step B.7 (page 62), and set them aside for step 8 or Part H (page 84).
6. Using a pipet, transfer a portion of tissue fraction SA2-L2 from step 4 to a conical GC vial. Place this vial under a gentle stream of nitrogen gas (piped through only CH_2Cl_2-washed Teflon, stainless-steel, or glass tubing), and slowly evaporate 1/2 of the solvent.

G. Fraction SA2-L2 from Tissue (continued)

7. Repeat step 6 until the entire contents of the tube have been transferred to the conical GC vial. The volume of the concentrated fraction should be ca. 0.1 mL.

8. Cap the vial, and label it. Proceed to GC Analysis (Section 12, page 85) with the labeled vial, plus the analyte-calibration solution vials from step 5 (page 83).

H. Fraction SA2-L2 from Liver Tissue - Note: This Part is for livers that are moderately to heavily contaminated with PCBs or DDTs.

1. Proceed as in steps G.1-2 (page 83), and in step G.5 above.

2. Transfer ca. 0.1 mL of Fraction SA2-L2 into a conical GC vial. Cap the vial and label it. Proceed to GC Analysis (Section 12, page 85) with the labeled vial, plus the analyte-calibration solution vials from step G.5 (page 83).

3. Place the remaining Fraction SA2-L2 in a regular GC vial, add "R" to the label, and store the vial in a freezer as a reserve.

I. Recycle of Column Packing - Note: When the column no longer maintains its calibration with azulene/perylene, recycle the packing according to Section 6, Part F (page 52).

SECTION 12

GC ANALYSIS

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GC ANALYSIS

A. Equipment List - Note: Wash the autosampler syringe thoroughly with CH_2Cl_2 before using.

Gas Chromatograph (GC), Hewlett-Packard model 5880A, including:

capillary column inlet system, in the No. 2 position

autosampler

cartridge tape unit

flame-ionization detector (FID), in the No. 1 position

electron-capture detector (ECD), in the No. 2 position

modifications: The graphite O-ring is placed around the injector insert instead of a Viton O-ring. A Viton O-ring is installed beneath the septum. A slot is cut in 2 ea 1 x l-inch, 1/32-inch thick aluminum plates so that they may be inserted from opposite sides around the injection port, just above the gas lines, and between the septum retainer assembly and the insert retainer assembly. A 1/16-inch tube is installed to blow compressed air gently onto the cooling fins.

Calibration Solutions

AH GC-calibration-check solution

PES GC-calibration-check solution

COP GC-calibration-check solution

Gas Cylinders and Apparatus

air, Ohio breathing air, CGA Grade E (or equivalent)

argon/methane, 95:5 (v:v)

helium, grade 4.5 (purified, > 99.995%)

hydrogen, grade 5 (ultra pure, > 99.999%)

nitrogen, grade 4.5 (purified, > 99.995%)

molecular sieve traps (1 for each gas cylinder), Hydro-Purge model ASC-1, Coast Engineering Laboratory, Gardena, California

regulators (1 for each gas cylinder), 2-stage

oxygen traps, J & W Scientific, Inc.

A. Equipment List (continued)

Solvents

CH_2Cl_2 and CH_3OH, as needed

Other Materials and Apparatus

GC column, J & W Scientific Inc., fused silica, DB-5, 30-m

diamond-tip etcher

2 ea ferrules, J & W Scientific Inc., 0.4-mm graphite, part no. 500-2004

jeweler's loupe, 10x

typewriter correction fluid (e.g., Wite-Out)

leak detector, Snoop, Nupro Co.

septum, Alltech Associates, 3/8-inch, blue, stock no. 6514

O-ring, Viton 0.208-inch ID, Parker Seal Co.

brush for cleaning fused-silica liner

glass wool, as needed

10-µL syringe, Hamilton model 701N

2-mL GC vials, Varian, part no. 96-000099-00

100-µl conical GC vials, Wheaton, part no. 986281

flowmeter suitable for all gases used

soap

1/8-inch OD copper tubing

Swagelok adapters, as needed

1/8-inch Swagelok tee

l/8-inch Swagelok connectors, as needed

B. Column Installation for FID Operation (continued)

1. Place a column on the rack holder in the GC oven so that one end faces the injector and the other faces the detector (FID).
2. Slide the column nut over the injector end of the column.
3. Using the diamond-tip etcher, score the column lightly about 1 cm from the column end, then snap off the column tip at the etched point.
4. Slide the ferrule over the inlet end of the column.
5. Etch the column again ca. 2 mm below the previously cut end, and snap off the 2 mm above the etched point.
6. Examine the end with the jewelers loupe; if it is not smooth (clean cut) and perpendicular to the column sides, repeat step 5.
7. Slide the column nut up the column until only 35 mm of column extends beyond the base of the ferrule.
8. With Wite-Out, place a white mark on the column even with the base of the column nut.
9. Slide the column into the injector, and tighten the nut by hand until the column is held lightly in place.
10. Adjust the column so that the white mark is again even with the base of the nut, then tighten the nut just sufficiently that no gas escapes when tested with a leak detector.
11. Tighten the nut an additional 1/4 turn.
12. Repeat steps 2-6, but on the detector end of the column.
13. Slide the column nut up the column until 57 mm of the column extends beyond the ferrule, then repeat step 8.
14. Slide the column into the FID, and tighten the nut by hand until the column is held lightly in place.
15. Repeat steps 9-10.

C. Column Installation for ECD Operation

1. Repeat steps B.1-12.
2. Slide the column nut up the column until only 47 mm of the column extends beyond the ferrule.
3. Repeat steps B.13-15, except use the ECD instead of the FID.

D. Injector Maintenance

1. Cool the injector and the oven to near room temp, then loosen the column nut at the injector block.
2. Disconnect the air from the autosampler, and remove the sample tray.
3. Make sure that the autosampler door is closed and the carrier gas pressure is released, then tilt the autosampler back.
4. If the gases are not already installed, proceed to Part E first. Otherwise, turn off the cooling air and the carrier gas ("carrier C/D" valve).
5. Remove the septum retaining nut. Discard the septum if it is worn. Check the O-ring, and replace it if it is worn or cracked.
6. Unscrew the lower injector cover, and withdraw the fused-silica liner.
7. Discard the O-ring. Using a small stiff brush, wash inside and outside of the liner with soap and water.
8. Flush the liner thoroughly with water.
9. Hold the liner with a clamp, and wash it with CH_3OH and then CH_2Cl_2.
10. After the liner has dried, use a pair of small forceps and a small glass rod to place a 5-mm, lightly-packed, glass-wool plug into the liner, and push it 35 mm below the septum end of the liner.
11. Install a new O-ring.
12. Slide the O-ring onto the injector liner, and place the liner into the injector, taking care to slide the column into the liner.

D. Injector Maintenance (continued)

13. Reattach the injector cover, replace the O-ring on top of the injector cover, and replace the septum on top of the O-ring.
14. Screw on the septum retaining nut, and adjust the cooling fins.
15. Turn on the cooling air, and retighten the column nut at the injector.
16. Adjust the carrier gas pressure to 20 psi.
17. Return the injector temperature to 300°C and the oven temperature to 180°C.

E. Installation of Gases for FID

1. Attach a 2-stage regulator to a full nitrogen cylinder.
2. Connect a molecular-sieve trap to the nitrogen regulator.
3. Connect the trap to the "Aux 2" (make-up gas connector) gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
4. Open the nitrogen-cylinder valve, and adjust the output pressure to 50 psi.
5. Check all connections of the nitrogen delivery system with leak detector, and tighten or replace any that leak.
6. Adjust the Aux 2 pressure to 30 psi on the gauge on the front panel of the GC.
7. Attach a 2-stage regulator to a full hydrogen cylinder.
8. Connect a molecular-sieve trap to the hydrogen regulator.
9. Connect the trap to the hydrogen gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
10. Open the gas cylinder valve, and adjust the output pressure to 55 psi.
11. Repeat step 5 with the hydrogen delivery system.

E. Installation of Gases for FID (continued)

12. Adjust the hydrogen pressure to 30 psi on the gauge on the front panel of the GC.
13. Attach a 2-stage regulator to a full helium cylinder.
14. Connect a molecular-sieve trap and an oxygen trap to the helium regulator.
15. Connect the trap to the "carrier C/D" gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
16. Open the helium-cylinder valve, and adjust the output pressure to 40 psi.
17. Repeat step 5 with the helium delivery system.
18. Adjust the carrier-gas pressure to 20 psi on the "carrier C/D" gauge on the front panel of the GC.
19. Attach a 2-stage regulator to a full air cylinder.
20. Connect a molecular-sieve trap to the air regulator.
21. Connect a 1/8-inch Swagelok tee to the outlet of the trap. Connect one end to the autosampler and the other end to the "air" gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
22. Open the air-cylinder valve, and adjust the output pressure to 75 psi.
23. Repeat step 5 with the air delivery system.
24. Adjust the air pressure to 30 psi on the gauge on the front panel of the GC.
25. Attach the high-flow line from the gas flowmeter to the split vent.
26. Adjust the split vent flow to 40 mL/min with the "C" flow valve.
27. Attach the low-flow line from the gas flowmeter to the septum purge vent.

E. Installation of Gases for FID (continued)

28. Adjust the septum purge flow to ca. 10 mL/min with the septum purge valve.
29. Make sure that the injector split vent flow is still 40 mL/min.
30. Detach both flowmeter lines.

F. Installation of Gases for ECD

1. Attach a 2-stage regulator to a full argon/methane cylinder.
2. Connect a molecular-sieve trap and an oxygen trap to the argon/methane regulator.
3. Connect the oxygen trap to the "Aux 2" (make-up gas) gas port in the back of the GC, using 1/8-inch OD copper tubing and Swagelok connectors.
4. Open the argon/methane cylinder valve, and adjust the regulator output pressure to 60 psi.
5. Adjust the "Aux 2" pressure to 30 psi on the gauge on the front panel of the GC.
6. Check all connections with leak detector, and tighten or replace any that leak.
7. Repeat steps E.13-23 and E.25-30 (pages 92 and 93), making certain that the air valve on the front of the GC remains turned off.

G. Entering and Storing the GC Program "ROUTINE" (explanatory notes on right margin in parentheses)

1. Press the CLEAR ENTRY button on terminal 1.
2. Press the ENTER button on terminal 1.
3. Type the following lines (letters will appear capitalized in the GC printout), and press the RETURN button after each line:

G. Entering and Storing the GC Program "ROUTINE" (continued)

```
 10 option base 1
 20 rem overnight sample runs
 25 gosub 1300                          (set up autosampler information)
 30 dim s(25)
 40 for i=1 to 25
 50 s(i)=0
 60 next i
 70 input "total number of samples to run",n    (enter the number of GC
                                                  vials to be analyzed)
 80 If n<26 then 110
 90 print "maximum of 25 samples allowed"
100 goto 70
110 input "enter starting bottle number",b
120 for i=1 to n                                (store vial nos. for
                                                  each sample)
130 s(i)=b
135 b=b+2
140 print "enter sample name for bottle #";s(i)    (enter sample name)
145 if i>14 then 151
150 on i goto 160,180,200,220,240,260,280,300,320,340,360,380,400,420
151 on i-14 goto 440,460,480,500,520,540,560,580,600,620,640
152 print "on goto error - line 150-151"
153 goto 2000
160 input a$
170 goto 660
180 input b$
190 goto 660
200 input c$
```

G. Entering and Storing the GC Program "ROUTINE" (continued)

```
210 goto 660
220 input d$
230 goto 660
240 input e$
250 goto 660
260 input f$
270 goto 660
280 input g$
290 goto 660
300 input h$
310 goto 660
320 input i$
330 goto 660
340 input j$
350 goto 660
360 input k$
370 goto 660
380 input l$
390 goto 660
400 input m$
410 goto 660
420 input n$
430 goto 660
440 input o$
450 goto 660
460 input p$
```

G. Entering and Storing the GC Program "ROUTINE" (continued)

```
470 goto 660
480 input q$
490 goto 660
500 input r$
510 goto 660
520 input s$
530 goto 660
540 input t$
550 goto 660
560 input u$
570 goto 660
580 input v$
590 goto 660
600 input w$
610 goto 660
620 input x$
630 goto 660
640 input y$
660 next i
670 input "which analysis file to use",z$          (get GC conditions
                                                    from Analysis File
680 execute x, "get analysis """&z$&""" device# 6"          on tape)
685 if x<>0 then 2000
690 For i=1 to n                                   (print sample name
                                                        on chart)
695 wait
696 list
700 print using 710; "sample:"
```

G. Entering and Storing the GC Program "ROUTINE" (continued)

```
710 image #,10/,5x,8a
720 image x,50a,2/
725 if i>14 then 731
730 on i goto 740,760,780,800,820,840,860,880,900,920,940,960,980,1000
731 on i-14 goto 1020, 1040,1060,1080,1100,1120,1140,1160,1180,1200
732 print "on goto error in line 730"
733 goto 2000
740 print using 720;a$
750 goto 1230
760 print using 720;b$
770 goto 1230
780 print using 720;c$
790 goto 1230
800 print using 720;d$
810 goto 1230
820 print using 720;e$
830 goto 1230
840 print using 720;f$
850 goto 1230
860 print using 720;g$
870 goto 1230
880 print using 720;h$
890 goto 1230
900 print using 720;i$
910 goto 1230
920 print using 720;j$
```

G. **Entering and Storing the GC Program "ROUTINE" (continued)**

```
930 goto 1230
940 print using 720;k$
950 goto 1230
960 print using 720;l$
970 goto 1230
980 print using 720;m$
990 goto 1230
1000 print using 720;n$
1010 goto 1230
1020 print using 720;o$
1030 goto 1230
1040 print using 720;p$
1050 goto 1230
1060 print using 720;q$
1070 goto 1230
1080 print using 720;r$
1090 goto 1230
1100 print using 720;s$
1110 goto 1230
1120 print using 720;t$
1130 goto 1230
1140 print using 720;u$
1150 goto 1230
1160 print using 720;v$
1170 goto 1230
1180 print using 720;w$
```

G. Entering and Storing the GC Program "ROUTINE" (continued)

```
1190 goto 1230
1200 print using 720;x$
1210 goto 1230
1220 print using 720;y$
1230 rem
1240 valve 6 on                                  (close inlet-purge valve;
                                                    inject next GC sample)
1250 execute x, "edit auto seq 8, "&val$(s(i)+1)
1260 if x<>0 then 2000
1270 start auto seq s(i),s(i)
1280 next i
1290 oven temp initial value 180
1300 execute x, "edit auto seq 1,2"
1310 if x<>0 then 2000
1320 execute x, "edit auto seq 2,0"
1330 if x<>0 then 2000
1340 execute x "edit auto seq 3,5"
1350 if x<>0 then 2000
1360 execute x, "edit auto seq 4,l"
1370 if x<>0 then 2000
1380 execute x, "edit auto seq 5,l"
1390 if x<>0 then 2000
1400 execute x, "edit auto seq 9,lO"
1410 if x<>0 then 2000
1420 return
2000 end
save prgm "routine" device# 6.
```

H. Entering/Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-1)

Note: The options for GC analysis files are:

HEXANE, for 1-mL GC/FID samples;

HEXANE EC, for GC/ECD samples, use brackets [ ];

HEXANE CON-1, for 0.1-mL GC/FID samples, use parentheses ( ).

1. Press the CLEAR ENTRY button on terminal 1.
2. Press the ENTER button on terminal 1.
3. Type the following lines (letter will appear capitalized in the GC printout), and press the RETURN button after each line.

```
oven temp limit 320
oven temp 50
oven temp on
det 1 temp limit 325          [det 2 temp limit 325)
det 1 temp 320                [det 2 temp 320]
det 1 temp on                 [det 2 temp on]
inj 2 temp limit 320
inj 2 temp 300
inj 2 temp on
detector b on                 [detector c on]
delete run tbl
run tbl on
run tbl annotation on
run time 0.50 valve 6 off
run time 80.0 stop            [run time 105.5 stop]
signal B device# 12           [signal c device# 12]
signal on device# 12
stop plot device# 12
chart speed 0.70 device# 12
```

H. Entering/Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-1) (continued)

```
attn 2↑2 device# 12          [attn 2↑8 device #12] (attn 2↑–1 device #12)
%offset 10 device# 12
zero on device# 12
intg signal B                [intg signal c]
sync off
run time annotation on
oven temp equib time 1.00
delete oven temp
oven temp initial time 3.00
oven temp 1 prgm rate 4.00
oven temp 1 final value 300  [oven temp 1 final value 170]
oven temp 1 final time 10.00 [oven temp 1 final time 0.00]
                             [oven temp 2 prgm rate 1.00]
oven temp annotation on      [oven temp 2 final value 210]
                             [oven temp 2 final time 0.00]
valve 6 off                  [oven temp 3 prgm rate 4.00]
                             [oven temp 3 final value 300]
peak width 0.04              [oven temp 3 final time 10.00]
threshold -3                 [threshold 6]
report on device# 0
report on
report annotation on
delete report tbl
report time 0.00 reject 1e+16
report time  1.00 bl mode 0
report time 5.00 reject 0.1 [report time 5.00 reject 100]
area%
delete calib
```

H. Entering and Storing Analysis File HEXANE [or HEXANE EC] (or HEXANE CON-1) (continued)

edit calib 0,1

edit calib -1,5

edit calib -2,5

edit calib -3,0

edit calib -4,""

edit calib -5,0

save analysis "hexane" device #6 [save analysis "hexane ec" device# 6]
(save analysis "hexane con-1" device# 6)

I. Verification of Stable GC Performance - Note: The options for analysis files:

HEXANE (for GC/FID check);

HEXANE EC (for GC/ECD check).

1. Place 6 vials containing the desired GC-calibration-check solution in the first 6 odd-numbered slots of the autosampler.
2. Place hexane-filled wash-vials in the even-numbered slots following each GC-calibration-check solution vial.
3. Press the GET and PRGM buttons, then type "routine" (including the quotation marks; letters will appear capitalized).
4. Press the following buttons: DEVICE# 6 and RETURN. After typing the correct response to each question that is asked, press the RETURN button. For analysis file option, see Note above.
5. Use the 6th GC injection to calculate the analyte peaks in the previous calibration runs as if they were unknowns (Part K, page 104).
6. The GC is operating properly if the deviation between calibrations is $\leq$ 5% for any analyte standard peak in calibrations #3-#6. If this criterion is not met, troubleshoot, adjust or repair the GC instrument, and repeat steps 1-5 until the criterion is met.

J. GC Analyses of Extract Fraction Concentrates

1. If there are n GC samples to be analyzed, let q be the next larger integer than [n/4 + 3]. Take 1 of the 2 analyte-calibration {AC) vials accompanying the fractions, and divide its contents into q subsamples in conical GC vials.
2. Label these AC conical (ACC) vials sequentially with the sample no. plus "-Al", "-A2", "-A3", etc. Similarly prepare 1 conical vial from the 2nd AC vial, and label it with the sample no. plus "-B". Cap these ACC vials, and use them to calibrate the GC for analysis of the corresponding set of sample extract fractions.
3. Load the GC-sample and ACC vials into the odd-numbered slots of the autosampler tray, as follows (column by column):

initial group	repeating group (as needed)	final group
hexane blank	(sample no.-A2)	sample no.-Aq-1
sample no.-B	(hexane blank)	hexane blank
sample no .-Al		sample blank
hexane blank	(sample no.)	sample no.
sample no. (1st)	(sample no.)	sample no.
sample no. (2nd)		sample spike
sample no. (etc.)		sample no.-Aq
sample no.		GC-calib.-check soln.

4. Place hexane-filled wash vials in the even-numbered slots.
5. Press the GET and PRGM buttons, then type: "routine" (including the quotation marks; letters will appear capitalized).
6. Press the following buttons: DEVICE#, 6 and RETURN. Type the correct response to each question asked, then press the RETURN button. For the analysis file option, see the Note in Part H (page 100).

K. GC Repeatability and Calibration Mixture Verification

To assess the repeatability, use the 3rd ACC vial (sample no.-A2) as the reference for calculating the relative responses of the other ACC vial analyses. Do this by calculating for each analyte the ratio of the response factor in an ACC analysis to that for the ACC reference analysis, and express the result as a percent. The response factor for an individual analysis is defined as R2/R3, using the definitions of R2 and R3 shown for Equation 12-1, page 108. If the ACC reference analysis is denoted with a "°", then the ratio of the response factors, expressed as a percent reduces to:

$$100 \times R_2^\circ \times R_3 \,/\, R_3^\circ \times R_2,$$

where the undenoted R_2 and R_3 stand for the corresponding parameters of the ACC analysis being compared to the ACC reference analysis. A deviation > 5% from 100% indicates a problem with the GC system (e.g., a leaking septum, a loose ferrule, or a worn out or dirty column). Such problems should be rectified before proceeding with analyses of the extract fractions.

To check the integrity of the solutions in the ACC vials, calculate for each analyte the ratio of the response factor for the GC-calibration-check solution to that of the last ACC vial (sample no.-Aq). Use the same formula given above, except the last ACC vial takes the place of the undenoted ACC vial and the GC-calibration-check solution vial takes the place of the ACC reference vial, with appropriate changes in the definitions of the R_2° and R_3° parameters (i.e., substitute "GC-calibration-check solution" for "ACC reference vial"). A deviation > 5% from 100% indicates a problem with the solution in the last ACC vial (sample no.-Aq), and perhaps with the other ACC vials also.

L. Analyte and I-Std Calculations

Identify the analyte peaks in the chromatograms of the extract fractions by comparing them with the analyte retention times obtained from the chromatogram of the ACC reference vial. Fractions analyzed by GC/ECD that show the presence of PCBs will have PCB peaks in addition to those corresponding to the PCB standards. Representative extracts need to be analyzed by GC/MS to identify these peaks and verify the other analytes indicated by retention time comparisons.

The GC/MS chromatograms are used to label the peaks in the GC/ECD chromatogram. Generally, the GC/MS is not as sensitive as the GC/ECD, so the fraction may need to be concentrated to as little as 20 µL for GC/MS analysis. Analyze a tenfold concentrated AC solution for the chlorinated compounds listed in Table 12-1. Determine the sum (Σ) of the selected ion areas (A) and the total ion current (TIC) for each analyte. Calculate the response ratio for each analyte standard by the equation: RR = TIC / CA.

For a multicomponent GC/MS peak, estimate the percent of each analyte using the areas of the selected ions indicated in Table 12-1. For example, for a 2-component GC peak containing analytes x and y (peak x+y), set the MS data system to determine the sum (Σ) of the ion areas (A) for analyte x (ΣA_x) and analyte y (ΣA_y). Calculate the percentage of x (% x) in peak x+y by the equation:

$$\frac{100(\Sigma A_x)(RR_x)}{(\Sigma A_x)(RR_x) + (\Sigma A_y)(RR_y)} = \% \text{ x.}$$

Then calculate % y by substituting (RR_y) for (ΣA_x)(RR_x) in the numerator.

L. Analyte and I-Std Calculations (continued)

Table 12-1. Selected ions to be used for estimating proportions of analytes in multicomponent GC/MS peaks.

Analytes	Selected Ions (m/z)
dichlorobiphenyls (set)	222,224
trichlorobiphenyls (set)	256,258,260
tetrachlorobiphenyls (set)	290,292,294,296
pentachlorobiphenyls (set)	324,326,328,330
hexachlorobiphenyls (set)	358,360,362,364,366
heptachlorobiphenyls (set)	392,394,396,398,400
octachlorobiphenyls (set)	426,428,430,432,434,436
nonachlorobiphenyls (set)	460,462,464,466,468,470
DDE's (set)	246,248
DDD's and DDT's (set)	235,237
trans-nonachlor	405,407,409,411,413
α-chlordane	371,373,375,377,379
aldrin	261,263,265,267
dieldrin	79
mirex	270,272,274,276
hexachlorobenzene	282,284,286,288
lindane (γ-BHC)	181,183,185
heptachlor	100
heptachlor epoxide	351,353,355,357
Internal Standards	
tetrachloro-m-xylene (TCMX)	242,244,246
dibromooctafluorobiphenyl (DBOFBP)	454,456,458

L. Analyte and I-Std Calculations (continued)

The peak areas of each PCB with the same number of chlorine atoms (isomer set) are to be summed to give the total area for that set (e.g., the dichlorobiphenyls). Because the calibration standard contains only one isomer for each set, use the response of that isomer as a surrogate standard to calculate the amounts of the other isomers in the set. In addition, report separately the concentration of each calibration isomer in each extract.

Note: All extracted sulfur (S_8) must be removed from the fractions before analysis for PCBs because S_8 interferes with the GC/ECD and GC/MS responses.

The I-Stds added to the sample at the beginning of the extraction are used to adjust for analyte losses during sample workup. Use Equation 12-1 (page 108) to calculate the analyte concentration in the sample on a dry weight basis. In the calculations for the AHs, use naphthalene-d_8 as the I-Std for naphthalene, 2-methylnaphthalene and 1-methylnaphthalene. Use perylene-d_{12} as the I-Std for benz[a]anthracene and the AHs below it in Table 2 (page 2). Calculate all other AH analytes in Table 2 using acenaphthene-d_{10} as the I-Std. To calculate the results for the chlorinated analytes, use dibromooctafluorobiphenyl (DBOFBP) as the I-std.

Use Equation 12-2 (page 109) to calculate the percent recovery of each I-Std. It involves the use of a GC/I-Std (HMB and/or TCMX) added to the extract fraction just before it is transferred to the GC vial. If < 50% of the I-Std is recovered, reanalyze the unused portion of the sample.

L. Analyte and I-Std Calculations (continued)

Equation 12-1, calculation of the concentration of an analyte in an aquatic sediment or tissue sample, dry weight basis:

$$\frac{\text{ng of the analyte}}{\text{g of the sample, dry wt}} = \frac{R_1 \times R_2}{R_3} \times \frac{\text{ng I-Std added to sample}}{\text{sample wt}} \times \frac{100}{\%\ \text{dry wt}^*},$$

where

$$R_1 = \frac{\text{analyte peak area from the analysis of the extract fraction}}{\text{I-Std peak area from the analysis of the extract fraction}},$$

$$R_2 = \frac{\text{analyte concentration in the ACC reference vial (ng/µL)}}{\text{I-Std concentration in the ACC reference vial (ng/µL)}}, \text{ and}$$

$$R_3 = \frac{\text{analyte peak area from the analysis of the ACC reference vial}}{\text{I-Std peak area from the analysis of the ACC reference vial}}.$$

* See Section 9 (page 65).

L. Analyte and I-Std Calculations (continued)

Equation 12-2, calculation of percent (%) recovery of internal standard (I-Std):

$$\% \text{ recovery of the I-Std} = \frac{R_1 \times R_2}{R_3} \times \frac{\text{ng GC/I-Std added to the fraction}}{\text{ng I-Std added to the sample}} \times 100 ,$$

where

$$R_1 = \frac{\text{I-Std peak area from the analysis of the extract fraction}}{\text{GC/I-Std peak area from the analysis of the extract fraction}} ,$$

$$R_2 = \frac{\text{I-Std concentration in the ACC reference vial (ng/µL)}}{\text{GUI-Std concentration in the ACC reference vial (ng/µL)}} , \text{ and}$$

$$R_3 = \frac{\text{I-Std peak area from the analysis of the ACC reference vial}}{\text{GC/I-Std peak area from the analysis of the ACC reference vial}} .$$

M. Spiked Blank Calculations

Identify the analyte peaks in the chromatograms of the spiked blanks by comparing them with the analyte retention times obtained from the chromatogram of the ACC reference vial. Calculate the percent (%) recovery of the analytes in the spiked blanks using Equation 12-3. Calculation of I-Std recovery is unchanged.

Equation 12-3, calculation of the percent recovery of analytes added to a blank sample:

$$\% \text{ recovery of analyte} = \frac{R_1 \times R_2}{R_3} \times \frac{\text{ng I-Std added to the blank sample}}{\text{ng analyte added to the blank sample}} \times 100,$$

where R_1, R_2 and R_3 correspond to the definitions given on page 108.

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APPENDIX

SUGGESTED ANCILLARY METHODS

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SUGGESTED ANCILLARY METHODS

The methods listed herein are additional procedures which NAF has recently adopted. Though they are built upon years of experience, they do not have the advantage of years of testing behind them. Thus, they are offered provisionally with the view that they may be of some use to those wishing even tentative recommendations. As with the Standard Procedures, we welcome suggestions and comments. At present these consist of convenient ways to prepare composite samples. No claims are made as to their statistical validity.

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1. Sediment Composites

A. Equipment and Materials List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample

untreated samples (as specified)

4-oz jar with cap

spatulas

Teflon sheeting (to line the jar cap)

labels

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Identify the sediment samples specified for preparing the composite sample. Remove the samples from the freezer and allow them to thaw completely.
2. Decant the standing water from the top of each sample. Using a spatula, stir each sample to homogenize thoroughly, and discard all pebbles, shells, biota, and other detritus.
3. Using a spatula, remove ca. 15 g of each sample and place it into the 4-oz jar. Stir the resulting mixture thoroughly to form a homogeneous composite. Return the unused portion of each sample to the freezer.
4. Cap the jar, label it with the appropriate composite sample designation, and store it in the freezer until needed.
5. Record in the log book the number of each sample used to prepare the composite sample.

2. Tissue Composites

A. Equipment and Materials List - Note: CH_2Cl_2-wash all glassware and materials contacting the sample

untreated samples (as specified)

2-oz bottle with cap

forceps

dissection scissors

spatulas, as needed

Tekmar Tissumizer

Teflon sheeting (to line the bottle cap)

500-mL Teflon wash-bottle (CH_2Cl_2-filled)

B. Procedure

1. Identify the untreated tissue samples specified for preparing the composite sample. Remove the samples from the freezer and allow them to thaw completely.
2. Using the forceps and scissors, remove ca. 1/2 of each sample and place it into the 2-oz bottle. Return the remaining half of each sample to its original container and store in the freezer.
3. If the combined weight of the sample portions forming the composite sample is < 10 g proceed to step 4. If the weight is > 10 g proceed to step 5.
4. Using a spatula, macerate and mix the composite in the 2-oz bottle until it is thoroughly homogenized.
5. Using the Tekmar Tissumizer, macerate and mix the composite in the 2-oz bottle for 1 min at a setting of 50.

B. Procedure (continued)

6. Cap the bottle, label it with the appropriate composite sample designation, and store it in the freezer until needed.
7. Record in the log book the number of each sample used to prepare the composite sample.